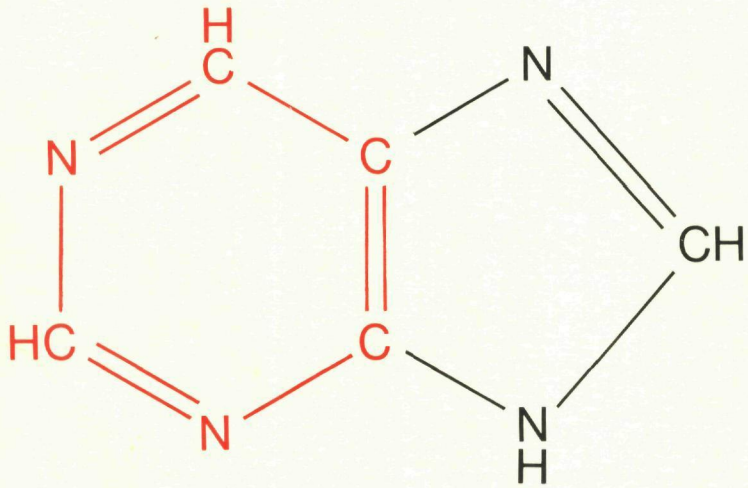
THE INTERRELATIONSHIP OF PYRIMIDINE AND PURINE METABOLISM IN MAMMALIAN BLOOD CELLS



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op gezag van de Rector Magnificus,
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volgens besluit van het College van Decanen
in het openbaar te verdedigen
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1978
Krips Repro Meppel

Aan de nagedachtenis van mijn moeder

Aan mijn vader

Voor Marijke

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A B B R E V I A T I O N S

ADA	adenosine deaminase
APRT	adenine phosphoribosyltransferase
2,3-DPG	2,3-diphosphoglycerate
EC	Enzyme Commission number
EDTA	ethylenediaminetetraacetate
EHNA	erythro-9-(2-hydroxy-3-nonyl)adenine
h(r)	hour
HGPRT	hypoxanthine-guanine phosphoribosyltransferase
K_i	inhibitor constant
K_m	Michaelis-Menten constant
MEMS	minimum essential medium for suspension cultures
min	minute(s)
MW	molecular weight
Na^+-K^+-ATPase	sodium plus potassium stimulated adenosine triphosphatase
ODC	orotidine 5'-monophosphate decarboxylase
OMP	orotidine 5'-monophosphate
OPRT	orotate phosphoribosyltransferase
PEI-cellulose	polyethyleneimine-cellulose
PNP	purine nucleoside phosphorylase
PRPP	5-phosphoribosyl 1-pyrophosphate
PRPP synthetase	ATP: ribose 5-phosphate pyrophosphotransferase
R 5-P	ribose 5-phosphate
S.D.	standard deviation
TLC	thin-layer chromatography
Tris	tris(hydroxymethyl)aminomethane
TTP	thymidine 5'-triphosphate

PREFACE

This thesis results from a research project on inherited disorders of pyrimidine and purine metabolism, and the influence of antimetabolites on this part of metabolism Up to a few years ago purine and pyrimidine metabolism used to be regarded as separate entities, and this view is reflected in the way most biochemical textbooks deal with this part of intermediary metabolism. Pyrimidine and purine metabolism, however, are closely interrelated. This interrelationship will be the main theme of this thesis, and is illustrated in three sections dealing with the role of phosphoribosylpyrophosphate (PRPP), the effects of the purine analog allopurinol on pyrimidine metabolism, and the possible disturbances of pyrimidine metabolism secondary to deficiency of the purine enzymes adenosine deaminase and purine nucleoside phosphorylase. The investigations were limited to mammalian blood cells and involved both intact cells and extracts of erythrocytes and lymphocytes.

Chapter 1 deals with our current knowledge of pyrimidine and purine metabolism, its regulation and the disturbances caused by enzyme deficiencies or enzyme inhibitors.

In chapters 2-5 the role of PRPP in pyrimidine and purine metabolism is investigated. First the enzymes which utilize PRPP in mammalian erythrocytes were studied (chapter 2). The assays developed to measure the concentration and synthesis of PRPP are described in chapter 3. They were used to study PRPP metabolism in hyperuricemia and gout (chapter 4) and to investigate the synthesis and degradation of PRPP in erythrocytes from ten mammalian species (chapter 5)

Chapter 6 deals with the effect of allopurinol administration on the urinary excretion of orotic acid and orotidine This effect can be ascribed to inhibition of orotate phosphoribosyltransferase and orotidine 5'-monophosphate decarboxylase, as shown in chapter 7 This chapter also describes the allopurinol-mediated stabilization of these enzymes in erythrocytes The stability of enzymes of pyrimidine metabolism in aging erythrocytes is the main subject of chapter 8. Chapter 9 deals with the effect of several purine and pyrimidine nucleotides on the activities of orotate phosphoribosyltransferase and orotidine 5'-monophosphate decarboxylase.

The activities of adenosine deaminase and purine nucleoside phosphoryl-

ase were determined in lymphocytes and erythrocytes of man, horse and cattle (chapter 10). The activity of the former enzyme was very low in horse lymphocytes. Since deficiency of adenosine deaminase in man seems to interfere with pyrimidine biosynthesis de novo, the relative contributions of the de novo and salvage pathways to the synthesis of pyrimidine nucleotides were evaluated for human and horse lymphocytes (chapter 11).

Finally, in chapter 12 the main results of the preceding chapters are discussed in order to demonstrate the relationships between pyrimidine and purine metabolism.

CHAPTER 1.

GENERAL INTRODUCTION

1.1. PYRIMIDINE METABOLISM

Pyrimidine nucleotides are precursors of nucleic acids and of nucleotide coenzymes involved in carbohydrate and lipid metabolism. The biological and clinical aspects of pyrimidine metabolism have been reviewed by Levine et al. (1). During normal cell growth and functioning an adequate supply of pyrimidine nucleotides is ensured by self-balancing networks of enzymatic reactions. In most cells from multicellular organisms, pyrimidine nucleotide biosynthesis can proceed by two mechanisms, de novo synthesis and the salvage pathway.

De novo synthesis of UMP requires six enzymatic steps (Fig. 1.1). The first step is catalyzed by carbamoylphosphate synthetase II (EC 2.7.2.9),

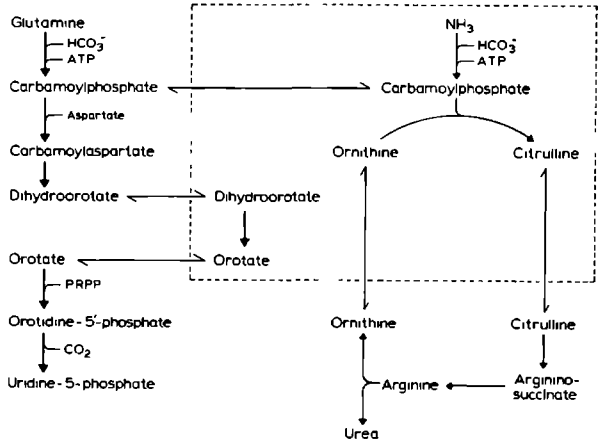

Fig. 1.1. The interrelation of pyrimidine biosynthesis de novo and the biosynthesis of urea. The dotted line represents the mitochondrial membrane.

a cytosol enzyme which utilizes glutamine as its nitrogen source. The enzyme is not present in mammalian erythrocytes (2). Carbamoylphosphate synthetase I (EC 2.7.2.5) which is located in the mitochondria of the liver utilizes ammonia and supplies carbamoylphosphate for the ornithine cycle. In hyperammonemia due to enzyme deficiencies of the ornithine cycle or induced by administration of ammonium ions (3) there is an increase in pyrimidine synthesis de novo indicating that intramitochondrial carbamoylphosphate is shunted from the ornithine cycle into pyrimidine synthesis. Recent evidence (4) suggests that also under normal conditions carbamoylphosphate synthetase I is an important source of carbamoylphosphate for pyrimidine synthesis in the liver. Aspartate transcarbamylase (EC 2.1.3.2) and dihydroorotase (EC 3.5.2.3) are the second and third enzyme of the de novo pathway and form together with carbamoylphosphate synthetase II a multifunctional enzyme complex (5,6). Dihydroorotate dehydrogenase (EC 1.3.3.1), the next enzyme in the pathway, is located on the mitochondrial inner membrane (7). The enzyme is not present in erythrocytes (2). The terminal two enzymes of the pathway, orotate phosphoribosyltransferase (OPRT, EC 2.4.2.10) and orotidine 5'-monophosphate decarboxylase (EC 4.1.1.23) are present in the cytosol as an enzyme complex (5). OPRT and ODC catalyze the conversion of orotate and phosphoribosylpyrophosphate (PRPP) to UMP and CO_2, with orotidine 5'-monophosphate (OMP) as an intermediate (Fig. 1.2). As inherited disorders of pyrimidine synthesis de novo orotic aciduria types I and II are known, which are caused by deficiency of OPRT and/or ODC (8).

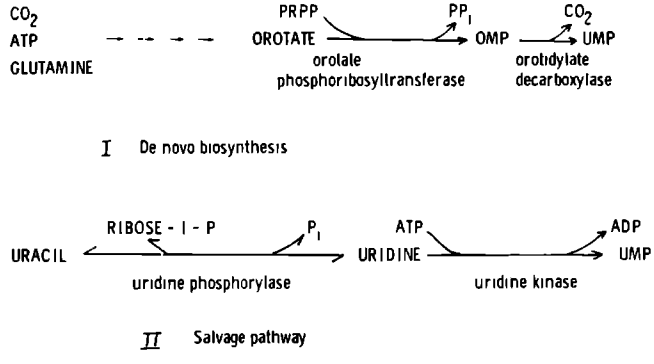

Fig. 1.2. Biosynthesis de novo and the salvage pathway for the synthesis of uridine monophosphate.

The salvage pathway for the synthesis of UMP (Fig. 1.2) involves the phosphorylation of uridine by uridine kinase (EC 2.7.1.48). Uridine may be derived from intracellular breakdown of nucleic acids. Cells may also use blood-borne nucleosides derived from dietary sources or synthesized in the liver by the de novo pathway (1). The reversible conversion of uridine to uracil is catalyzed by uridine phosphorylase (EC 2.4.2.3). The significance of the salvage pathway in relation to cell division and differentiation has been reviewed by Roux (9). Uridine kinase also phosphorylates a number of pyrimidine analogues. The chemotherapeutic aspects of this enzyme have been reviewed by Cihak and Rada (10).

Both de novo synthesis and the salvage pathway appear to be subject to feedback inhibition. UTP is an allosteric inhibitor of carbamoylphosphate synthetase II in mammalian cells (11). UTP and CTP inhibit purified uridine kinase. The inhibition appears to be competitive with respect to ATP (10). Uridine kinase was not inhibited, however, in intact cells which contained elevated concentrations of UTP or CTP (12). The regulatory role of PRPP in pyrimidine biosynthesis de novo is discussed in section 1.3.

In human erythrocytes a pyrimidine-specific 5'-nucleotidase is present. Deficiency of this enzyme is associated with hemolytic anemia (13).

1.2. PURINE METABOLISM

Purine nucleotides are precursors of nucleic acids and important sources of stored metabolic energy. The cyclic nucleotides cAMP and cGMP are involved in regulatory processes. ATP is thought to be a neurotransmitter in "purinergic" nerves. The significance of purine metabolism for cell physiology has extensively been reviewed by McKeran and Watts (14). The biosynthesis of purine nucleotides can be accomplished by de novo synthesis or by utilization of preformed purine bases or nucleosides (Fig. 1.3).

The de novo pathway is a sequence of ten enzymatic reactions leading to the synthesis of IMP. PRPP and glutamine are the substrates of the first enzyme, PRPP amidotransferase (EC 2.4.2.14). This enzyme is not present in mature erythrocytes (2,15). The synthesis of PRPP and its regulatory role are discussed in 1.3. IMP can be interconverted to AMP and GMP.

5'-nucleotidase (EC 3.1.3.5) catalyzes the cleavage of orthophosphate from the 5'-position of nucleoside monophosphates. The enzyme is deficient

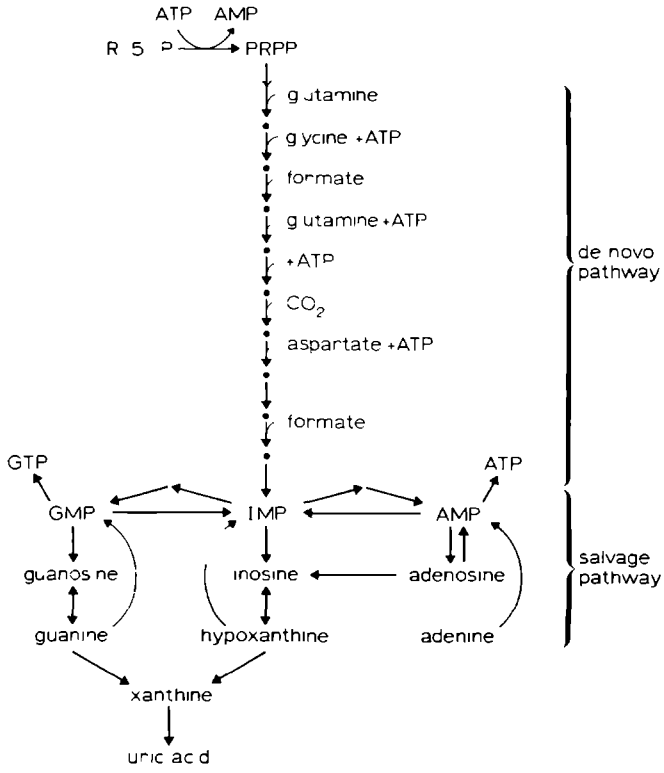

Fig. 1.3. De novo pathway and salvage pathway for the biosynthesis of purine nucleotides.

in lymphocytes of some patients with hypogammaglobulinemia (16). The nucleosides guanosine and inosine can be converted to guanine and hypoxanthine, respectively, in a reversible reaction catalyzed by purine nucleoside phosphorylase (PNP, EC 2.4.2.1). Deficiency of PNP is associated with defective T-lymphocyte function (17). Adenosine is not a substrate for PNP, but it can be deaminated by adenosine deaminase (ADA, EC 3.5.4.4). ADA deficiency is associated with a dysfunction of both B- and T-lymphocytes in one form of the severe combined immunodeficiency disease (18). The deficiencies of ADA and PNP will be discussed in more detail in 1.5.

Adenosine can be salvaged by phosphorylation to AMP, a reaction catal-

yzed by adenosine kinase (EC 2.7.1.20). The salvage of purine bases involves a reaction with PRPP to yield nucleotides, and is catalyzed by adenine phosphoribosyltransferase (APRT, EC 2.4.2.7) and hypoxanthine-guanine phosphoribosyltransferase (HGPRT, EC 2.4.2.8). The liver may be an important source of preformed purines which are then transported by erythrocytes to the peripheral tissues (19). The transfer of adenosine from rabbit liver to erythrocytes has been demonstrated (20). The salvage pathway is also important for the conversion of purine antimetabolites to their nucleotide derivatives, which in many cases are the active forms of the drugs (19). Enzyme deficiencies of both APRT and HGPRT have been described. Deficiency of APRT leads to accumulation of 2,8-dihydroxyadenine (21). HGPRT deficiency can lead to the Lesch-Nyhan syndrome or to X-linked gout (22-24). Hyperuricemia and hyperuricosuria are common to both diseases, but in the Lesch-Nyhan syndrome there are also neurological disturbances (compulsive self-mutilation, choreoathetosis).

Hyperuricemia may result from overproduction or underexcretion of uric acid. The renal contribution to hyperuricemia is outside the scope of this thesis and will not be discussed here. Excessive production of uric acid (primary metabolic gout) is usually associated with an accelerated rate of purine synthesis de novo. PRPP amidotransferase is the first and presumably rate-limiting enzyme of purine synthesis de novo. The activity of this enzyme is dependent on the concentrations of PRPP, purine ribonucleotides and orthophosphate (25-27). The role of PRPP is discussed in section 1.3. Purine ribonucleotides inhibit the purified enzyme (26), but enzyme activity in intact Ehrlich ascites cells was not inhibited even when purine nucleotide concentration was elevated threefold (28).

1.3. PRPP AND THE REGULATION OF PYRIMIDINE AND PURINE METABOLISM

PRPP plays a role in pyrimidine biosynthesis at two different sites. It is an allosteric activator of carbamoylphosphate synthetase II (11) and a substrate for OPRT (Fig. 1.4). The first enzyme of pyrimidine synthesis de novo appears to be rate-limiting (11). The conversion of orotate to OMP seems to be the second limiting step of the de novo pathway. In hyperammonemia, the increased availability of carbamoylphosphate for pyrimidine synthesis results in the accumulation of orotate (3). In HGPRT-deficient lymphoblasts

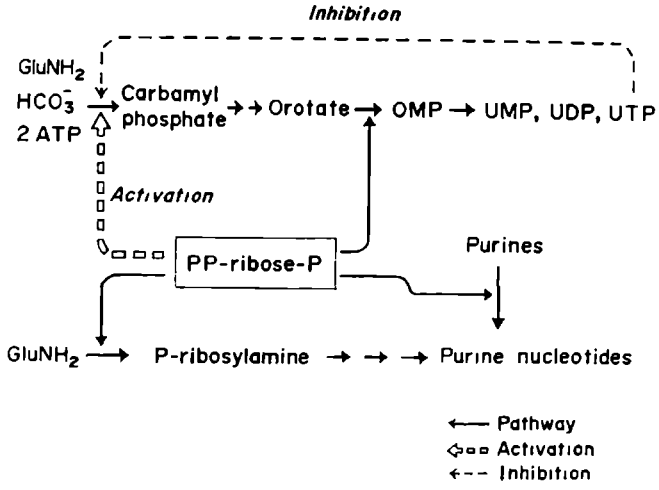

Fig. 1.4. The regulatory role of PRPP in the biosynthesis of pyrimidine and purine nucleotides. This figure is reproduced from Tatibana and Shigesada (11) with permission of the authors and publisher.

the increased intracellular PRPP concentration was associated with an increased concentration of pyrimidine nucleotides (29). This latter finding might also be explained by an activation of carbamoylphosphate synthetase II, but there was no evidence of an increased incorporation of [^{14}C]bicarbonate into pyrimidine nucleotides.

PRPP is also a substrate for the purine phosphoribosyltransferases (APRT and HGPRT) and for PRPP amidotransferase, and it converts the catalytically inactive form of PRPP amidotransferase to the active form (25). The intracellular concentration of PRPP is substantially lower than the K_m values of PRPP for the amidotransferase and HGPRT (30), and PRPP concentration may therefore be rate-limiting for these enzymatic reactions. The regulatory role of PRPP concentration with respect to purine biosynthesis de novo has been confirmed in experiments with intact Ehrlich ascites cells (28) and liver slices (31). Therefore an increased availability of PRPP may cause an accelerated rate of purine biosynthesis de novo and overproduction of uric acid. Hyperuricemia and gout associated with HGPRT deficiency can be ascribed to a reduced consumption of PRPP leading to a surplus in the amount available for purine synthesis de novo (30, 32). An excess of PRPP

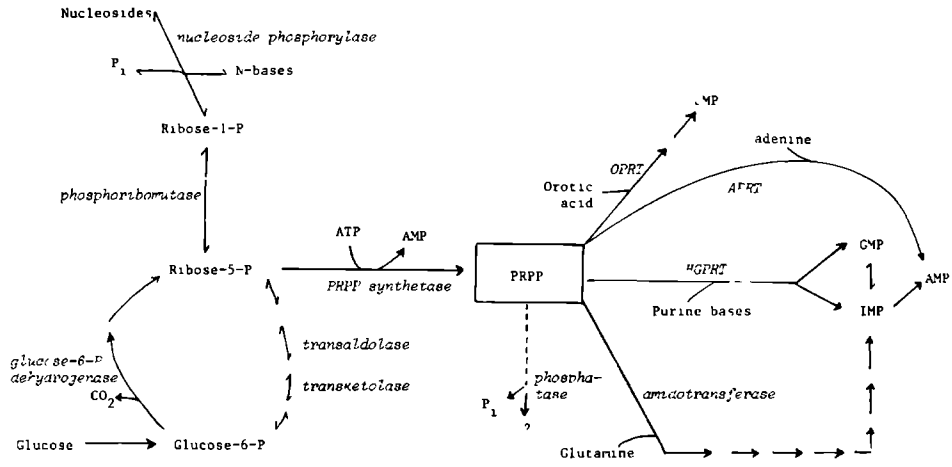

Fig. 1.5. Overall picture of the reactions involved in synthesis, utilization and degradation of PRPP.

can also result from hyperactivity of the enzyme PRPP synthetase (EC 2.7.6. 1). PRPP synthetase catalyzes the reaction between ATP and ribose 5-phosphate to form PRPP and AMP (Fig. 1.5). Orthophosphate is an essential allosteric activator of the enzyme (33, 34). Recent evidence indicates that PRPP synthetase is X-linked (35). Several mutations in this enzyme leading to purine overproduction have been found in gouty patients. The mutations may affect the regulatory site or the catalytic site of the enzyme. Sperling et al. (36) identified a mutant PRPP synthetase enzyme which had a decreased sensitivity to inhibition by AMP, ADP, GDP and 2,3-DPG. Becker et al. (37) discovered a mutant PRPP synthetase with a threefold increase in catalytic activity per enzyme protein molecule. Becker also reported the occurrence of kinetic variants of PRPP synthetase in some gouty patients. In these cases the enzyme purified from erythrocytes had a normal activity but an increased affinity for one of its substrates (38).

Ribose 5-phosphate, the precursor of PRPP, is a product of both the oxidative and the non-oxidative pentose phosphate pathways (Fig. 1.5). An alternative route is the phosphorolysis of ribonucleosides (39). The ribose

1-phosphate formed can be converted to ribose 5-phosphate by phosphoribomutase. In some gouty patients the excessive purine synthesis appeared to result from an increased ribose 5-phosphate concentration, leading to an increased availability of PRPP (38). An increased supply of ribose 5-phosphate may also contribute to the hyperuricemia associated with deficiency of glucose 6-phosphatase (32). The decreased concentration of PRPP in cultured lymphoblasts during glutamine limitation seems to result from a decreased availability of ribose 5-phosphate (39).

Degradation of PRPP by enzymatic hydrolysis occurs in homogenates of several human tissues (40). The hydrolysis does not require magnesium ions, in contrast to the utilization of PRPP by the pathways discussed above. Alkaline phosphatase appears to be the responsible enzyme.

1.4. EFFECTS OF ALLOPURINOL ON PURINE AND PYRIMIDINE METABOLISM

Allopurinol (4-hydroxypyrazolo (3,4-d)-pyrimidine) is a structural analog of hypoxanthine (Fig. 1.6). Its oxidation product, oxipurinol, closely resembles the pyrimidine compound orotic acid. Both allopurinol and oxipurinol can be converted to ribonucleotide derivatives, as shown in Fig. 1.7. They are mainly excreted as ribonucleosides. The metabolism of allopurinol and its effects on purine and pyrimidine metabolism have been reviewed (41).

Allopurinol and oxipurinol are potent inhibitors of xanthine oxidase (EC 1.2.3.2). Xanthine oxidase catalyzes the oxidation of hypoxanthine to xanthine and of xanthine to uric acid. Allopurinol has proven to be a suitable chemotherapeutic agent to reduce serum uric acid concentration in hyperuricemia. In most patients the replacement of urinary uric acid by the oxypurines, hypoxanthine and xanthine, is less than stoichiometric. This may indicate that de novo purine synthesis is suppressed by allopurinol, possibly by allopurinol 1-ribonucleotide (26). An alternative explanation would be that the oxypurines are not excreted but reutilized. Whatever the mechanism, this effect of allopurinol depends on HGPRT activity since in patients with HGPRT deficiency the oxypurine excretion increases during allopurinol therapy to the same extent as uric acid excretion decreases.

The administration of allopurinol also leads to interference with pyrimidine metabolism as indicated by an increased excretion of orotidine and orotic acid in urine (41). The increased excretion of orotidine can be

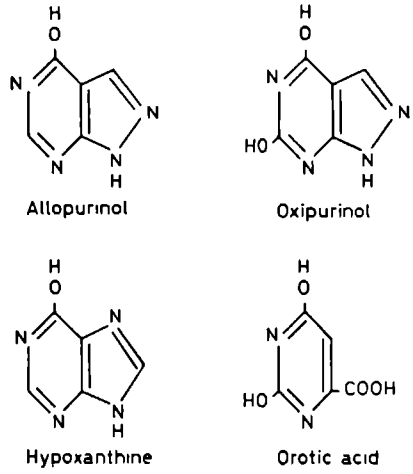

Fig. 1.6. Structural formulae of allopurinol, oxipurinol, hypoxanthine and orotic acid.

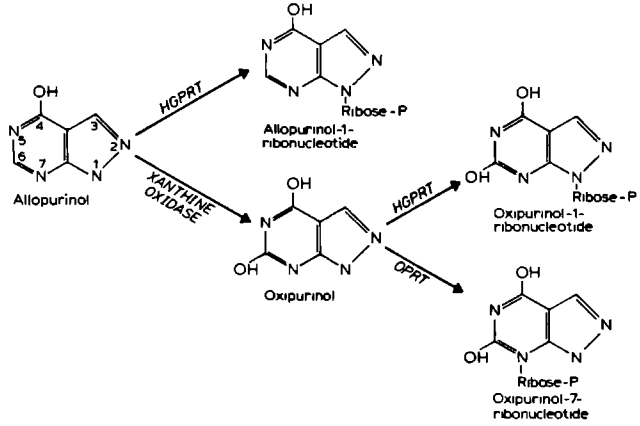

Fig. 1.7. The metabolism of allopurinol.

ascribed to inhibition of ODC by ribonucleotides of allopurinol and oxipurinol. The increased excretion of orotic acid suggested that allopurinol also causes inhibition of OPRT, but the mechanism of this inhibition had not yet been elucidated. Evidence will be presented in this thesis that the inhibition may be mediated by OMP. Allopurinol therapy also induces an apparent increase in the activities of OPRT and ODC in erythrocytes. Several hypotheses have been proposed to explain this phenomenon. Evidence presented in this thesis suggests that the apparent increase of enzyme activities is due to enzyme stabilization during the life-span of the erythrocytes. The effects of allopurinol on purine and pyrimidine metabolism and the presumable mechanisms of these effects are summarized in Table 1.1.

TABLE 1.1

EFFECTS OF ALLOPURINOL ON PURINE AND PYRIMIDINE METABOLISM

Effect	Mechanism	Effector
Hypouricemia	Inhibition of xanthine oxidase	Allopurinol Oxipurinol
Decreased purine biosynthesis	Inhibition of PRPP amidotransferase	Allopurinol-1-ribonucleotide?
Orotic aciduria	Inhibition of OPRT	OMP?
Orotidinuria	Inhibition of ODC	Oxipurinol-1-ribonucleotide Oxipurinol-7-ribonucleotide Allopurinol-1-ribonucleotide
Increased activity of OPRT and ODC in erythrocytes	Stabilization of OPRT and ODC	Allopurinol-1-ribonucleotide? Oxipurinol-7-ribonucleotide? OMP?

1.5. PYRIMIDINE METABOLISM AND THE DEFICIENCIES OF ADENOSINE DEAMINASE AND PURINE NUCLEOSIDE PHOSPHORYLASE

The association of severe combined immunodeficiency disease with deficiency of adenosine deaminase was first described by Giblett et al. (18). Subsequently, deficiency of purine nucleoside phosphorylase was detected in a

child with dysfunction of T-lymphocytes (17). The metabolic relationship of these sequential enzymes and the similar suppression of T-lymphocyte function suggest a common mechanism for impairment of the immune response. Furthermore, inosine which accumulates in PNP deficiency is a competitive inhibitor of ADA (42).

The precise mechanism by which these enzyme deficiencies are related to immunodeficiency is not known but several hypotheses have been proposed (Table 1.2). Most of these hypotheses are based on the assumption that ADA normally detoxifies adenosine by converting it to inosine which is then catabolized by PNP. Accumulation of adenosine or inosine may inhibit the uptake of other nucleosides into lymphocytes. Intracellular accumulation of adenine nucleotides may interfere with some essential process in the lymphocyte (e.g. synthesis of pyrimidine nucleotides, see below). The effects of adenosine on lymphoid cells have recently been reviewed by Seegmiller et al. (43). Another possibility is that a product formed by the action of ADA and PNP is essential for normal lymphocyte function. Finally, recent evidence suggests that the immunodeficiency may be caused by accumulation of deoxyribonucleotides (44, 45) leading to inhibition of the enzyme ribonucleotide reductase.

TABLE 1.2

POSSIBLE EFFECT OF ADA- OR PNP DEFICIENCY ON LYMPHOCYTES

I. Inhibition of uptake other nucleosides

II Intracellular accumulation of adenine nucleotides or cyclic AMP, which may cause.
   a inhibition PPRP synthetase by adenine nucleotides, by cyclic AMP or by depletion of P_i
   b. inhibition pyrimidine nucleotide synthesis de novo
   c. disturbance of energy charge
   d. inhibition of lymphocyte proliferation or transformation and cytotoxicity by cyclic AMP

III. Deficiency of products (inosine, hypoxanthine, ribose 1-phosphate)

IV. Trapping of purinedeoxyribonucleosides as phosphates. inhibition of pyrimidine deoxyribonucleotide synthesis.

Green and Chan (46) first suggested that ADA deficiency may cause an interference of pyrimidine biosynthesis de novo. The addition of adenosine to fibroblasts or lymphoid cells cultured in a medium free of ADA activity resulted in a depletion of cellular pyrimidine nucleotides and accumulation of orotic acid. These effects of adenosine could be prevented by uridine and were ascribed to an effect of adenine nucleotides on the activity of OPRT or the availability of PRPP (46, 47). Inhibition of cell growth, depletion of cellular pyrimidine nucleotides and accumulation of orotic acid were also found when lymphoma cells (48, 49) or lymphoblasts (50) were cultured in the presence of adenosine and erythro-9-(2-hydroxy-3-nonyl)-adenine (EHNA), an inhibitor of ADA. Again, uridine prevented the toxic effects of adenosine. The occurrence of orotic aciduria in two patients with deficiency of PNP (51) suggests that PNP deficiency also leads to interference with pyrimidine biosynthesis de novo.

Accumulation of orotic acid could be explained by a decreased intracellular concentration of PRPP. The content of PRPP in liver and spleen slices of the rat was markedly decreased by the addition of adenosine (52). Addition of adenosine in combination with the ADA inhibitor EHNA resulted

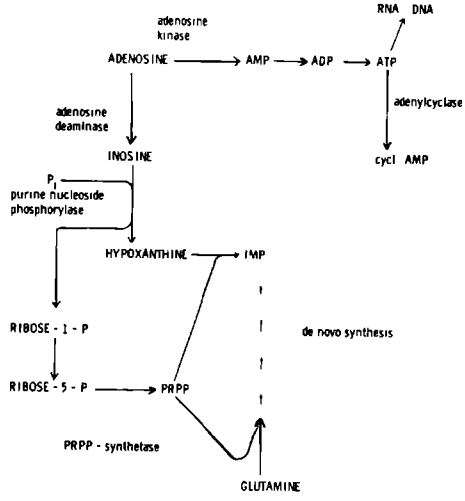

Fig. 1.8. The interrelation of adenosine metabolism and PRPP metabolism.

in a decreased PRPP concentration in lymphoma cells (49) and lymphoblasts (50). Inhibition of the enzyme PRPP synthetase by adenine nucleotides would offer an explanation for the decreased PRPP concentration. An alternative explanation would be a decreased availability of precursors of PRPP. Fig. 1.8 shows that the consecutive action of ADA and PNP on adenosine results in the production of ribose 1-phosphate, which can be converted to ribose 5-phosphate, the precursor of PRPP. The significance of PRPP for lymphocyte function is illustrated by the finding that an increase of the PRPP concentration is one of the early events in the stimulation of lymphocytes by phytohemagglutinin (53).

Whatever the mechanism of inhibition of pyrimidine biosynthesis de novo, it is obvious that the inhibition could be bypassed by means of the salvage pathway involving uridine.

1.6. AIM AND SCOPE OF THIS STUDY

The present investigation deals with the regulation of pyrimidine and purine metabolism, and the influence of inherited disorders and antimetabolites on this part of intermediary metabolism. In intact cells the pathways of pyrimidine and purine metabolism are not isolated entities but interact with each other and with the metabolism of amino acids and carbohydrates. Three aspects of the close interrelationship of pyrimidine and purine metabolism are investigated in detail.

First, special attention is given to the role of phosphoribosylpyrophosphate in pyrimidine and purine metabolism. The activities of the phosphoribosyltransferases (OPRT, APRT and HGPRT) in mammalian hemolysates are reported in chapter 2. In chapter 3 simple methods are described for estimating the concentration of PRPP and the activity of PRPP synthetase. These methods are used to investigate the role of PRPP in hyperuricemia and gout (chapter 4) and to measure the concentration, synthesis and degradation of PRPP in hemolysates from ten mammalian species (chapter 5).

Secondly, the effects of the purine analog allopurinol on pyrimidine metabolism are investigated. An isotope dilution assay is described which is adequate for the accurate and specific measurement of orotic acid and orotidine. This assay is applied to evaluate the effect of allopurinol administration on the urinary excretion of these compounds (chapter 6). In

chapter 7 mechanisms are proposed for the allopurinol-induced stabilization and inhibition of the enzymes OPRT and ODC. The stability of enzymes of pyrimidine metabolism in aging erythrocytes is the main subject of chapter 8. Chapter 9 deals with the effect of several purine and pyrimidine nucleotides on the activities of OPRT and ODC.

Thirdly, the possible interference of pyrimidine biosynthesis de novo secondary to deficiencies of enzymes of purine metabolism (ADA and PNP) is the starting-point for an investigation of pyrimidine metabolism in lymphocytes of man and horse. Horse lymphocytes are included in this investigation since ADA activity appeared to be virtually absent in these cells as reported in chapter 10. Chapter 11 deals with the relative contribution of the de novo pathway and the salvage pathway to the synthesis of pyrimidine nucleotides in lymphocytes of man and horse.

When studying metabolic pathways and their regulation, one must be very cautious in extrapolating the results of studies with cell extracts to the metabolism of intact cells or tissues. The conditions in which such studies are performed - high concentration of substrate, low concentration of enzyme and total protein, artificial ionic environment, etc. - are not at all like those that exist in intact cells. Furthermore, even if the results obtained with a cell extract reflect the intracellular metabolism, the conclusions drawn are not *a priori* valid for other cells than the particular type of cell that was studied. Whenever possible, the results obtained with cell extracts will be compared with measurements of the metabolism in intact cells. The red cell is a peculiar cell type since it contains no cell organelles and lacks several important enzymes of pyrimidine and purine metabolism (2). Therefore metabolic pathways are usually investigated both in erythrocytes and in leukocytes or lymphocytes.

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CHAPTER 2

ACTIVITY OF PURINE PHOSPHORIBOSYLTRANSFERASES AND OF TWO ENZYMES OF PYRIMIDINE BIOSYNTHESIS IN ERYTHROCYTES OF TEN MAMMALIAN SPECIES

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2.1. SUMMARY

1. Activities of HGPRT, APRT, OPRT and ODC have been determined in hemolysates from man and nine other mammalian species.
2. HGPRT was found to be nearly absent from horse erythrocytes, while low activities were found in dog and rat erythrocytes.
3. OPRT and ODC activities appeared to be very low in sheep erythrocytes, and below detection limit in horse erythrocytes.
4. OPRT and ODC activities were found to be coordinate in erythrocytes from all species studied.

2.2. INTRODUCTION

In our studies on the regulation of purine and pyrimidine metabolism we were looking for an animal which could serve as a suitable model for human purine and pyrimidine metabolism. In man, a deficiency of OPRT and ODC has been described, associated with orotic aciduria (Smith et al., 1961), while deficiency of APRT (Cartier and Hamet, 1974) and of HGPRT (Seegmiller et al., 1967) has been reported as well. HGPRT and APRT are enzymes of the purine salvage pathway. Some tissues have both salvage and *de novo* pathways for synthesis of purine nucleotides, but others have an absolute requirement for externally supplied purines. Erythrocytes may transport purines from the tissues where they are synthesized to their sites of utilization (Murray, 1971). HGPRT activity may play a role in purine transport across cell membranes (Benke et al., 1973). Because in most mammalian species little is known about the activity of these enzymes in erythrocytes, we decided to

determine the activities of HGPRT, APRT, OPRT and ODC in hemolysates from man and nine other mammals.

2.3. MATERIALS AND METHODS

Animals

Blood samples were taken from adult human volunteers or adult animals. Swiss mice (Mus musculus), Wistar rats (Rattus norvegicus), White New-Zealand rabbits (Oryctolagus cuniculus), Beagle dogs (Canis familiaris), Texel sheep (Ovis aries) and Rhesus monkeys (Macaca mulatta) were taken for blood samples. Blood samples from horse (Equus caballus), pig (Sus scrofa) and cattle (Bos taurus) were obtained from the local slaughterhouse.

Materials

Phosphorylribose-1-pyrophosphate (PRPP) tetrasodium salt was obtained from Boehringer, Mannheim. Orotic acid monosodium salt and orotidine 5'-monophosphoric acid trisodium salt were purchased from Sigma Chemical Co., St. Louis, U.S.A.

[Carboxyl-^{14}C]-orotic acid hydrate (42.4 mCi/mmole) and [carboxyl-^{14}C]-orotidine 5'-monophosphate triammonium salt (36.9 mCi/mmole) were obtained from New England Nuclear Corporation, as well as omnifluor and aquasol. [8-^{14}C]-Hypoxanthine (58 mCi/mmole) and[8-^{14}C]-adenine (54 mCi/mmole) were from the Radiochemical Centre, Amersham, England.

Aluminum sheets (20x20 cm) precoated with 0.1 mm of PEI-cellulose were obtained from Merck, Darmstadt, West Germany.

Preparation of hemolysates

Heparinized blood samples were centrifuged and erythrocytes were washed twice with 50 mM Tris-buffered saline (pH 7.4). Cells were lysed by addition of a 3-fold vol of 10 mM Tris buffer (pH 7.4). Some hemolysates, especially those from human volunteers and cattle, were prepared by freezing and thawing the cells twice in an aceton/dry ice bath.

Enzyme assays

Enzyme activities were estimated with radiochemical methods using ^{14}C-

labeled compounds. Determination of radioactivity was performed in a Packard 3380 liquid scintillation spectrophotometer with external standardization. Protein was determined according to Lowry et al. (1951). Specific enzyme activities are expressed in nmoles of product formed in 1 hr/mg of protein, at 37 ^{o}C and the assay conditions described below.

OPRT assay

The reaction mixture contained 0.3 mM [carboxyl-^{14}C]-orotic acid (0.15 mCi/mmole), 0.7 mM PRPP, 5 mM $MgCl_2$ and varying amounts of hemolysate protein (2-100 mg, depending on species studied) in 50 mM Tris buffer (pH 7.4). The final vol was 0.6 ml. Reactions were carried out in 30 ml-flasks sealed with rubber caps. Liberated $^{14}CO_2$ was trapped in 0.2 ml ethylene-glycol/ethanolamine (2:1, by vol), present in a small polypropylene tube fitted in a slightly larger tube. After incubation for 60 min with shaking OPRT activity was stopped by injection of 0.2 ml 0.25 M neutralized ethylene-diamine-tetraacetic acid. Because ODC does not require Mg^{2+}-ions decarboxylation can be allowed to proceed for another hour. Injection of 0.2 ml 5 M perchloric acid stops ODC activity and removes all $^{14}CO_2$ from the reaction mixture within one hour. The small tube containing trapped $^{14}CO_2$ is transferred to a scintillation vial with 10 ml of toluene-methanol (2.1, by vol) containing 4 g omnifluor per liter.

ODC assay

Hemolysate, appropriately diluted with 50 mM Tris buffer (pH 7.4) was incubated with 0.1 mM [carboxyl-^{14}C] orotidine 5'-monophosphate (0.2 mCi/mmole) for 60 min in a final vol of 0.55 ml. Reaction was terminated by injection of 0.2 ml 5 M perchloric acid. Production of $^{14}CO_2$ was measured as described above.

HGPRT assay

The activity of this enzyme was only measured with hypoxanthine as a substrate. Incubation mixture contained 125 mM Tris buffer (pH 7.4), 10 mM $MgCl_2$, 1 mM PRPP, 0.15 mM [8-^{14}C]-hypoxanthine (58 mCi/mmole) and 10-800 µg hemolysate protein in a final vol of 40 µl. In some experiments inosine was added to a final concentration of 1 mM. After incubation for 30 min reaction was terminated by immersing the tubes for 2 min in boiling water

and addition of excess carrier hypoxanthine and inosine. After centrifugation separation of nucleotides from nucleosides and purine bases was achieved by chromatography on PEI-cellulose plates according to Reyes (1972). Spots corresponding to IMP, inosine and hypoxanthine were localized under u.v. light, cut out from the thin-layer and eluted with 1 ml of 0.1 N HCl/0.2 M KCl in scintillation vials. After addition of 10 ml of aquasol radioactivity was determined.

APRT assay

The assay conditions were the same as for HGPRT, except that the reaction mixture contained 0.1 mM [8-^{14}C]-adenine (54 mCi/mmole) and 15-250 µg protein. After termination of the reaction carrier adenine and adenosine were added.

2.4. RESULTS AND DISCUSSION

Assays of enzyme activities were always performed with two different protein concentrations and established to be linear with time during incubation. Doubling the concentration of the substrates or the cosubstrate PRPP did not result in an increase of the enzyme activity. Values measured for enzyme activities of most mammals were not significantly affected by the choice of lysis procedure, whether the hypotonic shock or the freeze/thawing procedure was employed. Bovine and human erythrocytes, however, showed a relatively strong resistance against osmotic lysis and were lysed by freeze/thawing.

OPRT, ODC, HGPRT and APRT are present in hemolysates from all species studied with exception of horse erythrocytes, having OPRT and ODC activities below detection limit (Table 2.1).

OPRT and ODC activities were very low in sheep erythrocytes and showed considerable variation between individual animals. In all other species studied more precise measurements of OPRT and ODC activities could be made. These enzymes showed a coordinate relationship. ODC activity was about twice as high as OPRT activity in mammalian erythrocytes. Only pig and rabbit erythrocytes showed a different ODC/OPRT ratio. A coordinate relationship between OPRT and ODC was previously reported by Fox et al. (1971) for human erythrocytes and by Pausch et al. (1972) for various rat tissues.

TABLE 2.1.

ACTIVITY OF HGPRT, APRT, OPRT AND ODC AND ODC/OPRT RATIO IN MAMMALIAN ERYTHROCYTES

Enzyme activities are given in nmoles hr per mg protein. The results represent the mean ± S. Numbers in parentheses refer to the number of individuals.

Species	HGPRT	APRT	OPRT	ODC	ODC/OPRT
Man	9[illegible] 5 ± 8 3 (4)	17 0 ± [illegible] 0 (4)	0 [illegible] ± 0 08 (6)	1 [illegible] ± 0 0 (6)	[illegible] ± 0 [illegible] (6)
Monkey	31 3 ± 8 1 (10)	40 [illegible] ± 8 1 (5)	0 36 ± 0 17 (6)	1 [illegible] ± 0 40 (6)	[illegible] 3 ± 0 7 (6)
Dog	2 63 ± 1 06 (4)	3 8[illegible] ± 1 0[illegible] (9)	0 3[illegible] ± [illegible] 09 (9)	0 68 ± 0 18 (9)	C ± 0 [illegible] (9)
Mouse	9 59 ± 0 90 (5)	13 3 ± 3 1 (5)	0 50 ± 0 04 (5)	0 9[illegible] ± 0 08 (5)	1 9 ± 0 2 (5)
Rat	3 24 ± 0 36 (4)	3 [illegible]1 ± 0 50 (5	0 [illegible]1 ± 0 06 6)	0 [illegible]7 ± 0 06 (6)	2 3 ± 0 4 (6)
Cattle	27 0 ± 4 [illegible] (5)	3 [illegible]6 ± 1 1[illegible] (5)	11 6 ± 1 2 (5)	2 [illegible] ± 1 7 (5)	9 ± 1 3 (5)
Rabbit	82 7 ± [illegible] 0 (4)	1 [illegible] ± [illegible] 1 (4)	5 08 ± [illegible] [illegible] (6)	5 86 ± [illegible] 1[illegible] (6)	1 [illegible] ± 0 2 6)
Pig	59 9 ± [illegible] 7 (4)	3 0[illegible] ± 0 81 (4)	0 32 ± 0 (10)	4 20 ± 0 60 (10)	13 8 ± 3 1 (10)
Sheep	53 0 ± 10 [illegible] (10)	[illegible] 46 ± 1 07 (10)	0 0[illegible]6 ± 0 01[illegible] (5)	0 035 ± 0 015 (5)	-
Horse	0 59 ± 0 11 (4)	13 8 ± 3 1 7)	<0 [illegible]05 (4)	<0 005 (4)	

OPRT and ODC activities measured in human erythrocytes are intermediate between the values reported by Fox et al. (1971) and those of Beardmore et al. (1972). Hatfield & Wyngaarden (1964) measured OPRT activity in sonified beef erythrocytes, freed from stroma by centrifugation. They reported a value of 11 nmoles/hr per mg protein.

The assay of purine phosphoribosyltransferase activities may be disturbed by 5'-nucleotidase (E.C.3.1.3.5), which converts the nucleotides formed to nucleosides. We found that no adenosine was formed during the APRT assay. However, small but significant amounts of inosine were formed from IMP under the conditions of HGPRT assay by lysates from human, rat and dog erythrocytes. Phosphorolytic cleavage of inosine by purine nucleo-

side phosphorylase (E.C.2.4.2.1) can occur in mammalian hemolysates (Duhm, 1974). When this occurs hypoxanthine is formed, the starting compound for HGPRT assay. Because of the existence of this cyclic pathway the measured activity of HGPRT under these conditions is a minimum value. Therefore we studied the effect of excess non-radioactive inosine on the value of HGPRT measured. Excess inosine will inhibit the cleavage of radioactive inosine formed via IMP from [8-^{14}C]-hypoxanthine. On the other hand, if the activity of purine nucleoside phosphorylase is very high, a significant amount of nonradioactive hypoxanthine is formed resulting in dilution of the radioactive substrate for HGPRT. Under reaction conditions employed, the sum of products (IMP plus inosine) was not significantly affected by the presence of excess inosine. This finding indicates that the cyclic pathway described above probably does not play a significant role. This may be caused by the low activity of 5'-nucleotidase in erythrocytes (Adams et al., 1971). One should be aware, however, of the possibility that in other tissues, especially when 5'-nucleotidase activity is high, the cyclic pathway described can interfere with HGPRT assay.

Xanthine oxidase (E.C.1.2.3.2) could also interfere with the HGPRT assay. This enzyme, however, is not found in mammalian blood cells (Al-Khalidi & Chaglassian, 1965). Chicken erythrocytes do contain xanthine oxydase which might be responsible for the low HGPRT activity found in these cells (Partsch & Altmann, 1973).

There seems to be no direct correlation between HGPRT and APRT activities in mammalian erythrocytes. No such correlation is found when comparing enzyme activities in different tissues of man (Rosenbloom et al., 1967) or Rhesus monkey (Krenitsky, 1969). In erythrocytes, however, HGPRT consumes a great deal of the available PRPP and deficiency of HGPRT is associated with increased PRPP concentration (Greene & Seegmiller, 1969). There is evidence that PRPP stabilizes the activity of the APRT enzyme (Greene et al., 1970). So HGPRT activity might have an indirect relationship to APRT activity in erythrocytes (Gordon et al., 1974). The PRPP concentration in mammalian erythrocytes and its possible correlation with activities of HGPRT and APRT is currently under investigation.

Purine phosphoribosyltransferase activities are also present in erythrocytes from mouse and rat. Murray (1966) reported absence of HGPRT from mouse and rat erythrocytes, while only traces of APRT activity were found. Adenine and hypoxanthine of low specific activities were used while incu-

bations were carried out for 4 min at 25 °C. In our experiments purine bases of high specific activity were used and up to 400 µg of hemolysate protein (for rat) had to be added in order to measure accurately HGPRT activity. Activities measured under these conditions are about an order of magnitude lower than those found by Murray in extracts from rat tissues.

HGPRT values are extremely low for horse erythrocytes. This low activity seems to be a genuine property of horse erythrocytes since intracellular IMP production was also very low when intact horse erythrocytes were incubated with [8-^{14}C]-hypoxanthine in phosphate buffer (pH 7.4), containing glucose and Mg^{2+} ions. HGPRT activity in horse leukocytes was about 30 nmoles/hr per mg protein, so the low HGPRT activity appears to be restricted to the erythrocytes. Horse erythrocytes may provide an animal model for studying some aspects of HGPRT deficiency. A complete deficiency of HGPRT is associated with the Lesch-Nyhan syndrome (Seegmiller et al., 1967), while a partial deficiency is seen in a subgroup of gouty patients (Kelley et al., 1969). There is, however, no clear-cut correlation between the severity of clinical manifestations and the severity of HGPRT deficiency measured in erythrocytes (Emmerson & Thompson, 1973). Dancis et al. (1973) have stressed the superiority of the leukocyte over the anucleate erythrocyte in providing an index of the enzymatic performance of other tissues.

Values for HGPRT in human erythrocytes (measured with hypoxanthine as a substrate) range from 71 $\pm$ 9 nmoles/hr per mg protein (Fox et al., 1971) to 128 $\pm$ 9 nmoles/hr per mg protein (Sorensen, 1970). For APRT in human erythrocytes, mean values from 14 to 35 nmoles/hr per mg protein (Watts et al., 1974, Bashkin et al., 1973) have been reported. Simmonds et al. (1973) reported a HGPRT activity of 54.8 nmoles/hr per mg protein in pig erythrocytes while for APRT a mean value of 4.3 nmoles/hr per mg protein was found. Krenitsky (1969) reported activities of 88 nmoles/hr per mg protein for HGPRT and 97 nmoles/hr per mg protein for APRT in extracts from blood cells of Rhesus monkey. These last values are much higher than the ones we have measured, which may be caused by the difference in preparation.

It was suggested that erythrocytes function as purine carriers (Murray, 1971). HGPRT and APRT may also have a role in the transport of purines across the cell membrane (Benke et al., 1973; De Bruyn & Oei, 1974). The low activities of APRT or HGPRT (or both) in some mammalian species do not support an involvement of these enzymes in purine transport in these animals. It is possible, however, that transport mechanisms for purines are different

between mammalian species.

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CHAPTER 3

A SIMPLE AND SENSITIVE METHOD FOR ESTIMATING THE CONCENTRATION AND SYNTHESIS OF 5-PHOSPHORIBOSYL 1-PYROPHOSPHATE IN RED BLOOD CELLS.

W.J.M.Tax and J.H.Veerkamp

(Clin.Chim.Acta (1977) 78, 209-216)

3.1. SUMMARY

A method is presented for the determination of 5-phosphoribosyl 1-pyrophosphate (PRPP), which is based on the release of $^{14}CO_2$ from [carboxyl-^{14}C]orotic acid by the consecutive action of orotate phosphoribosyltransferase and orotidine 5'-monophosphate decarboxylase. The assay is simpler and less timeconsuming than most methods currently employed and is equally sensitive. The method proved to be suitable for measuring low concentrations of PRPP such as found in human erythrocytes and fibroblasts.

An increased PRPP concentration was observed in erythrocytes from patients with partial or complete deficiency of hypoxanthine-guanine phosphoribosyltransferase, from some (but not all) gouty patients and from a patient with deficiency of purine nucleoside phosphorylase. PRPP synthetase activity was measured with a method similar to the assay for PRPP. In erythrocytes with an increased PRPP concentration, PRPP synthetase activity was found to be normal at both optimal and suboptimal substrate concentrations.

3.2. INTRODUCTION

5-Phosphoribosyl 1-pyrophosphate (PRPP) is a substrate common to several metabolic pathways including the biosynthesis of purine and pyrimidine nucleotides and the salvage pathway for purines. A number of studies (1,2) have stressed the importance of PRPP concentration with regard to the regulation of purine nucleotide synthesis de novo. An increased intracellular concentration of PRPP may be caused by increased synthesis (3,4) or decreas-

ed utilization (3,5) and can be involved in the pathogenesis of hyperuricemia.

In the present communication we describe a simple and rapid procedure for measuring low concentrations of PRPP such as found in human erythrocytes and fibroblasts. The assay is based on the release of $^{14}CO_2$ from [carboxyl-^{14}C]orotic acid in the presence of a mixture of orotate phosphoribosyltransferase (EC 2.4.2.10, OPRT) and orotidine 5'-monophosphate decarboxylase (EC 4.1.1.23, ODC). The OPRT-ODC reaction has been used before (6,7) to measure PRPP concentration in erythrocytes with a spectrophotometric technique but this method has not found much application. One reason for this might be that some investigators found no linear response at low PRPP concentrations (8). We have studied the validity of the radiochemical assay based on the OPRT-ODC reaction and found it to be reliable and linear with respect to PRPP concentration even at concentrations as low as 0.2 nmol per ml reaction mixture. In addition, in our hands this method was less time-consuming and more sensitive than techniques involving the chromatography of nucleotides formed in the presence of adenine phosphoribosyltransferase (EC 2.4.2.7) or hypoxanthine-guanine phosphoribosyltransferase (EC 2.4.2.8, HGPRT).

The OPRT-ODC catalyzed reaction was also used to measure PRPP synthetase (EC 2.7.6.1) activity. With this method we investigated whether the increase in erythrocyte PRPP concentration found in some patients was due to an increased PRPP synthesis. This investigation included a search for kinetic variants of PRPP synthetase. Becker (3) has reported the occurrence of kinetic variants of this enzyme in erythrocytes from some patients with hyperuricemia. This enzyme abnormality is not detected by standard screening techniques.

3.3. MATERIALS AND METHODS

Materials

Ribose 5-phosphate monobarium salt, PRPP tetrasodium salt and a preparation from brewers' yeast containing OPRT and ODC were obtained from Boehringer, Mannheim. [carboxyl-^{14}C]Orotic acid, omnifluor and aquasol were purchased from New England Nuclear Corp., Dreieichenhain, G.F.R. [8-^{14}C]Hypoxanthine was from the Radiochemical Centre, Amersham.

Preparation of hemolysate

Venous blood samples were obtained from six children with deficiency of hypoxanthine-guanine phosphoribosyltransferase exhibiting the Lesch-Nyhan syndrome (9), from a gouty adult (E.F.) having partial deficiency of HGPRT, from two gouty adults with normal activity of HGPRT, and from a patient with deficiency of purine nucleoside phosphorylase (EC 2.4.2.1) (10). Healthy adult volunteers served as control group. Blood was collected in heparinized tubes. Erythrocytes were isolated by centrifugation and washed twice with 50 mM Tris/HCl (pH 7.4) containing 100 mM NaCl. Lysates were obtained by freezing and thawing the red blood cells twice. Both PRPP and PRPP synthetase from human erythrocytes are stable during this treatment. However, PRPP synthetase from erythrocytes of several mammalian species is unstable and must be assayed in freshly prepared hemolysates obtained by hypotonic shock (details will be published elsewhere).

PRPP: assay I

The assay was performed in scintillation vials sealed with rubber caps. Hemolysate was diluted 25-fold with 10 mM Tris/HCl (pH 7.4) containing 1 mM EDTA. 1-ml samples of diluted hemolysate were kept in boiling water for 45 sec and immediately chilled on ice. During this procedure all interfering enzyme activities were destroyed while most of the PRPP remained intact. A correction was made for destruction of PRPP by measuring the recovery of PRPP (3.5 nmol), added to the hemolysates prior to the heating step. After thorough mixing, 100 µl of a suspension containing 0.4 mg (3 mU) of the OPRT-ODC preparation in 100 mM $MgCl_2$ was added. Reaction was started by addition of 30 nmoles of [carboxyl-^{14}C]orotic acid (10 mCi/mmol). Liberated $^{14}CO_2$ was trapped in 0.2 ml ethyleneglycol/ethanolamine (2 . 1, by vol.), present in a small polypropylene tube fitted in a slightly larger tube. After shaking for 15 min at 37 ^{o}C, 0.2 ml of 1 M HCl was injected and shaking was continued for 1 h to remove all $^{14}CO_2$ from the reaction mixture. The small tube containing trapped $^{14}CO_2$ was transferred to a scintillation vial and 10 ml of toluene/methanol (2 : 1, by vol.) containing 4 g omnifluor per liter was added. Blanks contained Tris buffer (10 mM, pH 7.4) instead of hemolysate.

PRPP. assay II

This assay is based on the HGPRT-catalyzed production of [^{14}C]IMP (inosine 5'-monophosphate) from [^{14}C]hypoxanthine. HGPRT was partially purified from human erythrocytes by using its heat stability. 2 ml packed cells were diluted 10-fold with distilled water and heated for 1 min at 100 °C. Precipitate was removed by centrifugation. Heating and centrifugation were repeated once and the resulting colorless supernatant was lyophilized. The residue was taken up in 1 ml of 10 mM Tris/HCl (pH 7.4). The assay mixture contained in a final volume of 40 µl: 5 µmol Tris/HCl (pH 7.4), 0.4 µmol $MgCl_2$, 8 nmol [8-^{14}C]hypoxanthine (58 mCi/mmol), 10 µl of the crude HGPRT suspension and 1-3 nmol of PRPP. After 15 min at 37 °C reaction was stopped by immersing the tubes in boiling water for 5 min. Carrier hypoxanthine and inosine (50 nmol of each) were added and reaction mixtures centrifuged. Hypoxanthine, inosine and IMP were separated on polyethyleneimine-cellulose, localized with ultraviolet light and eluted with 0.1 M HCl/0.2 M KCl according to Reyes (11). After addition of 10 ml of aquasol radioactivity was determined. Results of PRPP measurements are expressed in nmol per ml packed cells.

PRPP synthetase

Reaction was carried out in scintillation vials sealed with rubber caps. Reaction mixture contained 25 mM phosphate buffer (pH 7.4), 1 mM ATP, 1 mM ribose 5-phosphate, 10 mM $MgCl_2$, 0.3 mM [carboxyl-^{14}C]orotic acid (3 mCi/mmol), 0.4 mg of the OPRT-ODC preparation and 0.5-1 mg of hemolysate protein. When enzyme activity was measured at suboptimal substrate concentration, the concentration of ATP or ribose 5-phosphate was decreased to 20 µM while the concentration of the other substrate was maintained at 1 mM. $^{14}CO_2$ produced was trapped in ethanolamine as described above. Incubation was stopped after 30 min at 37 °C with injection of 0.2 ml 1 M HCl. Shaking was continued for 1 h and $^{14}CO_2$ measured as described above. Enzyme activity is expressed as nmol per h per mg protein. Protein was determined according to Lowry et al. (12).

The validity of the OPRT-ODC based assay of PRPP was ascertained by measuring various concentrations of PRPP using both the method based on the OPRT-ODC reaction and the technique involving the HGPRT-catalyzed conversion of [^{14}C]hypoxanthine to [^{14}C]IMP. Fig.3.1 shows that the results from both methods closely agree. Furthermore, a linear relationship was found between the concentration of PRPP present and the amount of $^{14}CO_2$ released, even at concentrations as low as 0.2 nmol per ml of reaction mixture. During the heating step some PRPP is destroyed but the percentage of loss is reproducible and does not depend upon PRPP concentration (Fig.3.2). The mean recovery with 13 hemolysates was 82 $\pm$ 4%. So a correction can be made for the percentage lost during heating.

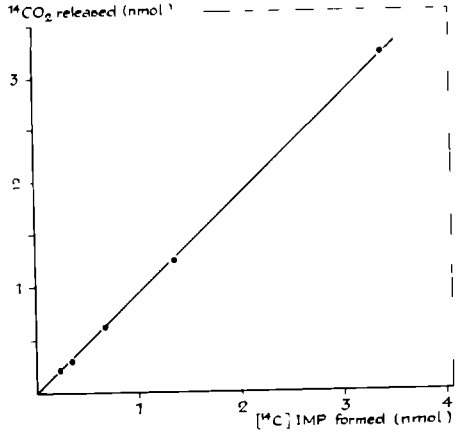

Fig. 3.1.
Relationship between the amount of PRPP present and the amount of $^{14}CO_2$ released from [carboxyl-^{14}C]orotic acid. The amount of PRPP was assayed by measuring the production of [^{14}C]IMP from [^{14}C]-hypoxanthine by partially purified HGPRT, or the release of $^{14}CO_2$ as described in Materials and Methods except that the heating step was omitted.

When EDTA was absent during the heating step, a low recovery of PRPP was observed (Table 3.1). This phenomenon was not due to inhibition of the OPRT-ODC reaction but was the result of excessive destruction of PRPP. This destruction is not caused by an enzymatic process since it is not prevented by denaturation of the hemolysate protein. As a matter of fact, denaturation results in an even lower recovery of PRPP. Addition of Mg^{2+} ions apparently stimulates an enzymatic breakdown of PRPP. Evidence will be presented elsewhere that acid phosphatase may be the enzyme responsible.

PRPP concentration was measured in erythrocytes of normal adults and of a group of patients having a metabolic disorder related to purine

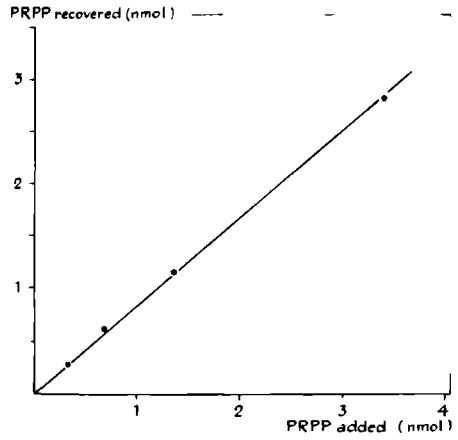

Fig. 3.2.
Recovery of various amounts of PRPP added to hemolysates. The amount of PRPP added was measured with the HGPRT-based assay. After heating in boiling water for 45 sec PRPP recovery was assayed by measuring the release of $^{14}CO_2$ from [carboxyl-^{14}C]orotic acid. A correction was made for PRPP present in the hemolysate.

TABLE 3.1.

STABILITY OF PRPP DURING HEATING

3.5 nmol of PRPP was added to 1 ml hemolysate (15 mg protein) and heated in boiling water for 45 sec in the presence or absence of 1 mM EDTA and 10 mM $MgCl_2$ as indicated. Denatured hemolysate protein was obtained by heating the hemolysate in boiling water for 5 min prior to the addition of PRPP. Results are expressed as the percentage of the amount of PRPP added. Data are the means of three experiments.

Conditions of heating			PRPP recovery (%)
Hemolysate protein	EDTA	Mg	
Native	+	–	82
Native	–	–	23
Denatured	–	–	8
Native	+	+	39
Denatured	+	+	76

TABLE 3.2.

ERYTHROCYTE PRPP CONCENTRATION IN SOME PATIENTS WITH A DISORDER IN PURINE METABOLISM

Subject	Pathology	PRPP (nmol/ml packed cells)
Re W	HGPRT deficiency	28.0
Ru W		33.3
P v d A		44.0
Ph v d A		26.0
P d R		21.8
L.K		38.2
L.F	Partial HGPRT deficiency	6.5
R V	Purine nucleoside phosphorylase deficiency	5.9
L.N W	Gout	3.6
E v d W	Gout	4.0
H V	Gout	1.2
Controls (n = 6)	–	15 ± 0.2

metabolism (Table 3.2). Partial deficiency of HGPRT was associated with an increased concentration of PRPP. A marked increase of PRPP concentration was observed in erythrocytes from six patients with complete deficiency of HGPRT showing the clinical features of the Lesch-Nyhan syndrome. A significant increase of erythrocyte PRPP concentration was also observed in a patient with deficiency of purine nucleoside phosphorylase. PRPP concentration was normal in erythrocytes from some patients with gout but increased in erythrocytes from two of these patients (L.N.W. and E.v.d.W.).

The possible involvement of increased synthesis of PRPP in the observed increase of PRPP concentration was investigated by measuring PRPP synthetase activities. The enzyme preparation from erythrocytes requires phosphate for its activity. A concentration of 20 mM was found to be saturating. Michaelis-Menten kinetics were observed with respect to ATP and ribose 5-phosphate. K_M values were 59 and 60 μM, respectively (each value is the mean of three experiments). At high concentrations of ribose 5-phosphate (> 0.5 mM) the Lineweaver-Burk plot deviates from linearity, suggesting substrate inhibition. The kinetic data were used to select optimal and suboptimal substrate conditions. Measurements were then made in hemolysates from controls and from some patients with increased erythrocyte PRPP concentration (Table 3.3). PRPP synthetase was not significantly increased in erythrocytes of any of the patients studied, either at optimal or at suboptimal conditions.

TABLE 3.3.

PRPP SYNTHETASE ACTIVITY IN HUMAN HEMOLYSATE AT OPTIMAL AND SUBOPTIMAL SUBSTRATE CONCENTRATIONS

Subject	Activity (nmol/h per mg protein)		
ATP	1 mM	1 mM	20 μM
Ribose 5-P	1 mM	20 μM	1 mM
Controls (n = 5)	45.0 ± 2.7	11.9 ± 0.7	12.9 ± 2.0
HGPRT deficient			
Ru W	37.0	11.8	15.7
Re W	40.4	12.5	13.9
Purine nucleoside phosphorylase deficient			
R V	38.6	8.6	11.6
Gouty			
L.N W	46.0	11.9	10.1
E. v d W	38.8	10.7	11.5

3.5. DISCUSSION

The OPRT-based assay of PRPP described above includes a heating step which prevents interference of the assay by enzyme activities present in the hemolysates tested (e.g. PRPP synthetase and PRPP-degrading enzymes). The assay is simpler and less time-consuming than procedures involving chromatographic or electrophoretic analysis of small samples from the incubation mixture. Furthermore, since one of the reaction products is measured completely and the blank value does not depend on the amount of incubation mixture which is analysed, but remains low, a high signal to noise ratio can be obtained by increasing the amount of hemolysate in the incubation mixture.

The assay responds linearly over the concentration range tested, from 0.2 to 3.5 nmol of PRPP per ml of reaction mixture (Fig.3.1). Johnson et al. (8) have stated that no such linearity was found at PRPP concentration lower than 10 nmol per ml. The discrepancy might be related to a difference in technique since we used a radiochemical procedure instead of a spectrophotometrical one. The importance of the presence of EDTA during extraction of PRPP from the cells is stressed by the results given in Table 3.1. Heavy metals apparently stimulate the destruction of PRPP. Denaturation of hemolysate protein may increase the concentration of heavy metals by releasing them from metallo-proteins.

Normal values for erythrocyte PRPP concentration obtained with this technique are similar to values reported by most other investigators (3, 13, 14). Methods that do not involve destruction of PRPP synthetase activity by heating seem to result in higher values for normal erythrocyte PRPP concentration (4, 15, 16). Other investigators previously observed also an increase of PRPP concentration in erythrocytes from patients with partial (17) or complete (3, 5, 17) deficiency of HGPRT.

The rate of synthesis of PRPP is normal in erythrocytes from patients with deficiency of HGPRT. An increased PRPP synthetase activity in cultured lymphocytes (18) and fibroblasts (19) from patients with deficiency of HGPRT has been associated with the increased PRPP concentration. However, other investigators have found normal activities of PRPP synthetase in HGPRT-deficient lymphoblasts (20) and fibroblasts (21). Anyway, in HGPRT-deficient erythrocytes the increase of PRPP concentration is not caused by an increased activity of PRPP synthetase and results from a decreased utilization of PRPP. The increased PRPP concentration observed in the patient with

deficiency of purine nucleoside phosphorylase is not the result of increased PRPP synthetase activity, as is evident from Table 3.3. Since deficiency of purine nucleoside phosphorylase blocks the synthesis of hypoxanthine from inosine, the activity of HGPRT might be markedly decreased due to lack of substrate and the increased PRPP concentration may be ascribed to a decreased utilization of PRPP.

No increase of PRPP synthetase was found in erythrocytes from two gouty patients despite an increased PRPP concentration in their erythrocytes. Enzyme activity was also normal at suboptimal ATP or ribose 5-phosphate concentration indicating normal kinetics. The increase of PRPP may be associated with a decreased utilization instead of an increased production. This possibility is currently under investigation.

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CHAPTER 4

CONCENTRATION AND SYNTHESIS OF PHOSPHORIBOSYLPYROPHOSPHATE IN ERYTHROCYTES OF GOUTY PATIENTS AND ADOLESCENTS WITH A HIGH SERUM URATE CONCENTRATION

4.1. SUMMARY

Hyperuricemia may result from overproduction of phosphoribosylpyrophosphate (PRPP), a key substrate of purine biosynthesis. The occurrence of inborn abnormalities of the enzyme PRPP synthetase was investigated in erythrocytes of a group of 50 adolescents with a high concentration of urate in serum and in a group of 12 adults with primary gout. The concentration of PRPP in erythrocytes was normal in all subjects. No striking increase of PRPP synthetase activity was found in any subject, but the group of adolescents with a high serum urate concentration had a significantly higher activity of PRPP synthetase than the control group. No kinetic variants were detected when enzyme activity was measured at non-saturating concentrations of the substrates ATP or ribose 5-phosphate. The results are discussed in relation to the late onset generally observed in gout.

4.2. INTRODUCTION

Uric acid is the end product of purine metabolism in man. Gout is a disease associated with hyperuricemia and results from the deposition of urate crystals and tissue reactions to the crystals. It is manifested clinically as arthritis, tophi and/or urate nephropathy. In primary gout there is a hereditary disorder leading to increased production of uric acid (primary metabolic gout) or decreased excretion of this compound (1,2).

The availability of phosphoribosylpyrophosphate (PRPP) plays an important role in the regulation of purine biosynthesis de novo (3-5), and an excess of PRPP leads to purine overproduction and hyperuricemia (2,3).

Deficiency of hypoxanthine - guanine phosphoribosyltransferase (HGPRT, EC 2.4.2.8) results in a decreased utilization of PRPP and an increased concentration of this compound (2,3). A larger availability of PRPP may also be caused by an increased synthesis. Becker et al. (6) and Sperling et al. (7,8) found abnormal forms of the enzyme PRPP synthetase in some gouty patients, which were associated with an increased catalytic activity per enzyme protein molecule (6) or a decreased sensitivity to feedback inhibition (7,8). The enzyme mutations were unique among the inborn errors of metabolism in causing hyperactivity rather than deficiency of the enzyme. Meyskens and Williams (9) found a normal PRPP concentration and PRPP synthetase activity in erythrocytes from 7 patients with primary gout and from 10 adult patients (mean age 49 years) with primary hyperuricemia. Feedback inhibition of the enzyme by ADP, GDP and 2,3-DPG was also normal. We now measured the concentration and synthesis of PRPP in erythrocytes of 12 gouty patients and of 50 adolescents having a high concentration of serum urate. This investigation included a search for kinetic variants of the enzyme since Becker (10) reported that some gouty patients have an increased affinity of PRPP synthetase for one of the substrates. This enzyme abnormality is not detected under standard assay conditions. Earlier (11) we reported an increased PRPP concentration in erythrocytes of 2 gouty patients. PRPP synthetase activity was normal, however, also at suboptimal conditions. PRPP concentration was normal in erythrocytes from one other gouty patient.

4.3. MATERIALS AND METHODS

Venous blood samples were obtained from 13 healthy male controls (22-30 years), from 12 adult male gouty patients (29-74 years) and from 50 adolescents (6 girls and 44 boys, mean age 18 years, range 10-23 years) with serum urate concentration higher than the 95th percentile point. Uric acid in serum of adolescents was determined by Dr. F.Klein (Faculty of Medicine, Erasmus University, Rotterdam) by a uricase-peroxidase procedure (12). Blood samples were transported in the presence of ice. Preliminary experiments had shown that both PRPP concentration and PRPP synthetase activity are stable for several hours under these conditions. Assays for PRPP and PRPP synthetase were based on the release of $^{14}CO_2$ from [carboxyl-^{14}C]orotic acid and were performed as previously described (11). When PRPP synthetase

activity was measured at suboptimal substrate concentrations, the concentration of ATP or ribose 5-phosphate was decreased to 50 µM while the concentration of the other substrate was maintained at 1 mM. These conditions yielded enzyme activities which were about half-maximal for hemolysates of controls. Protein was determined according to Lowry et al. (13). Statistical analysis was performed using the paired one-tailed Student's t-test.

4.4. RESULTS

The serum urate concentration of the group of adolescents included in the present investigation ranged from 399 to 553 µmol/l. Mean ± S.D. was 424 ± 31 µmol/l. The upper limit of normal is usually defined as 7 mg per 100 ml or 416 µmol/l (1). Since the various therapeutic treatments of the gouty

TABLE 4.1.

PRPP CONCENTRATION AND PRPP SYNTHETASE ACTIVITY IN ERYTHROCYTES OF CONTROLS, GOUTY PATIENTS AND ADOLESCENTS WITH A HIGH SERUM URATE CONCENTRATION

	PRPP	PRPP synthetase		
		ATP	1 mM	1 mM	50 µM
		Ribose-5-P	1 mM	50 µM	1 mM
Controls (n=10)	1.28 ± 0.41 (0.58 - 1.77)	49.7 ± 7.6 (40.4 - 64.5)	58 ± 4 (53 - 63)	51 ± 5 (44 - 58)
Gouty patients (n=12)	1.25 ± 0.41 (0.62 - 2.06)	53.7 ± 10.2 (38.9 - 69.4)	52 ± 7 (43 - 62)	52 ± 3 (48 - 57)
Adolescents (n=50)	0.91 ± 0.27 (0.45 - 1.49)	55.9 ± 5.9 (44.2 - 68.7)	55 ± 5 (40 - 64)	52 ± 5 (45 - 61)

The results represent the mean ± S.D. The range is given in parentheses. Concentration of PRPP is given in nmol/ml packed cells. Its concentration was measured in 13 control subjects. PRPP synthetase activity at optimal substrate concentrations is expressed as nmol/hr per mg protein. Enzyme activity at suboptimal substrate concentrations is expressed as a percentage of the activity measured at optimal substrate concentrations.

patients may markedly influence the serum urate concentrations, these concentrations are not given here.

The concentration of PRPP in erythrocytes of all subjects was within 2 S.D. of the mean value for the control group. The mean value for PRPP concentration was in the group of adolescents with a high serum urate concentration slightly below the mean of the controls (Table 4.1).

The mean activity of PRPP synthetase in the group of gouty patients was higher than in the control group but the difference did not achieve statistical significance. The activity of PRPP synthetase was significantly higher ($p < 0.01$) in the group of adolescents with a high serum urate concentration. Four subjects of the latter group and 2 gouty patients had PRPP synthetase activities which were more than 2 S.D. above the normal mean, although the enzyme activity never exceeded the normal mean + 3 S.D. The activity of PRPP synthetase in the group of adolescents did not correlate with the serum urate concentration. The affinity of the enzyme for the substrates ATP and ribose 5-phosphate was normal in all subjects studied. An increased substrate affinity would be reflected by an increased percentage of the maximal enzyme activity when the assay is performed with suboptimal substrate concentration.

5.4. DISCUSSION

The results presented here suggest that large disturbances in PRPP metabolism are not a major cause of hyperuricemia and gout. PRPP concentration was normal in erythrocytes of all subjects, in contrast to the increased concentration previously found (11) in 2 gouty patients. Meyskens and Williams (9) also found no increased PRPP concentration in erythrocytes from gouty patients or hyperuricemic subjects. Becker (10) found normal erythrocyte PRPP concentrations in several gouty patients with an increased PRPP concentration in fibroblasts. Furthermore, erythrocyte PRPP concentration may be affected by drug therapy (3). Measurement of erythrocyte PRPP concentration is therefore of limited value in the study of abnormalities in PRPP metabolism.

Hyperactivity of PRPP synthetase as reported by Becker et al. (6) and Sperling et al. (7) was not found in erythrocytes of any of the subjects studied. Fox (14) and Sperling et al. (15) reached a similar conclusion

after studying large numbers of hyperuricemic subjects, Meyskens and Williams (9) found a normal enzyme activity and normal feedback inhibition of PRPP synthetase in hyperuricemic and gouty patients. The results obtained by Becker (10), however, suggested that in a significant number of gouty patients PRPP synthetase has an abnormally high substrate affinity, but a normal activity at high substrate concentration. No such kinetic variants were detected in the present investigation. Abnormal kinetics of HGPRT may also be associated with hyperuricemia and gout (10). Although we did not investigate the kinetics of HGPRT, the activity of this enzyme is probably normal since we found a normal PRPP concentration in erythrocytes of all subjects studied. Partial (11,16) or complete (3,10, 11,16) deficiency of HGPRT leads to an increased PRPP concentration in erythrocytes.

The activity of PRPP synthetase in the group of adolescents with high urate concentration was significantly higher than in controls. When the one-tailed t-test is applied to the figures given by Sperling et al. (15) for gouty patients and controls, PRPP synthetase activity is also significantly higher in his group of 34 gouty patients ($p < 0.05$) although the authors state that there is no significant difference. Our small group of 12 gouty patients and the group of 7 gouty patients of Meyskens and Williams (9) did not show a significant increase. Primary gout usually manifests between the third and fourth decade of life, although it is regarded as a hereditary disorder (1). The late onset of the disease may be due to a slow accumulation of uric acid. A small but significant increase of the PRPP synthetase activity might therefore be of physiological significance. It seems worthwhile to investigate whether a similar increase in PRPP synthetase activity can be demonstrated in other cell types of patients with hyperuricemia. If available, liver cells would be more appropriate than erythrocytes to study the enzymatic basis of uric acid overproduction, since in man purine synthesis de novo takes place mainly in the liver. Cultured fibroblasts appeared already more useful than erythrocytes for classifying patients with uric acid overproduction and aberrations in PRPP metabolism (10).

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CHAPTER 5

PHOSPHORIBOSYLPYROPHOSPHATE IN ERYTHROCYTES OF TEN MAMMALIAN SPECIES: CONCENTRATION, SYNTHESIS AND DEGRADATION

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5.1. SUMMARY

1. The concentration of PRPP and the activity of PRPP synthetase have been measured in hemolysates from man and nine other mammalian species. PRPP synthetase activity was very low in dog hemolysate.
2. High concentrations of PRPP appeared to be associated with low levels of HGPRT activity, suggesting that HGPRT is the major pathway for utilization of PRPP in mammalian erythrocytes.
3. An alternative catabolic route for PRPP was observed in mammalian hemolysates, which seemed to be associated with acid phosphatase activity. The activity of acid phosphatase in mammalian hemolysates was measured.

5.2. INTRODUCTION

Previously we reported the activities of three phosphoribosyltransferases (HGPRT, APRT and OPRT) in erythrocytes of ten mammalian species (Tax et al., 1976). Enzyme activities showed large variations between species and it was speculated that the activities of HGPRT and/or APRT might be correlated with the concentration of PRPP. PRPP is a substrate common to several metabolic pathways including *de novo* synthesis of purine and pyrimidine nucleotides and the salvage pathways for purines. The intracellular concentration of PRPP regulates the rate of purine nucleotide synthesis *de novo* in nucleated cells (Kelley et al., 1970; Bagnara et al., 1974). The present communication deals with the results of our studies on PRPP in mammalian erythrocytes. The actual PRPP concentrations were measured as well as the activity of PRPP synthetase, the enzyme responsible for the synthesis of

PRPP. During these investigations we found evidence for a catabolic pathway for PRPP, which was not related to phosphoribosyltransferase activity. The enzymatic hydrolysis was stimulated by Mg^{2+} and may be due to acid phosphatase activity. Therefore, acid phosphatase activity was also determined in hemolysates of all mammals studied.

5.3. MATERIALS AND METHODS

Materials

Carboxyl-[^{14}C]orotic acid hydrate (42.4 mCi/mmole) and aquasol were obtained from New England Nuclear Corporation, 8-[^{14}C]inosine (60 mCi/mmole) from the Radiochemical Centre, Amersham, England. Ribose 5-phosphate, PRPP, acid phosphatase and a preparation from brewer's yeast containing OPRT and ODC were purchased from Boehringer, Mannheim. Ouabain and p-nitrophenyl-phosphate, disodium salt, were from Merck, Darmstadt, West Germany.

Hemolysates

Heparinized blood samples were obtained from the same mammalian species as were used in our previous investigations (Tax et al., 1976). Erythrocytes were obtained by centrifugation and washed twice with 50 mM Tris-HCl (pH 7.4) containing 100 mM NaCl. Cells were lysed by hypotonic shock with a 3-fold vol of 10 mM Tris-HCl (pH 7.4) or by freezing and thawing the cells twice in an aceton/dry-ice bath.

PRPP concentration, synthesis and hydrolysis

The assay of PRPP concentration is based on the release of $^{14}CO_2$ from carboxyl-[^{14}C]orotic acid by the consecutive action of purified OPRT and ODC. Synthesis of PRPP from ATP (1 mM) and ribose 5-phosphate (1 mM) in the presence of 20 mM P_i (pH 7.4) and 10 mM $MgCl_2$ was measured at 37 ^{o}C by trapping the $^{14}CO_2$ released from carboxyl-[^{14}C]orotic acid in the presence of OPRT and ODC. The details of both assays have been reported elsewhere (Tax & Veerkamp, 1977).

Hydrolysis of PRPP was measured by incubating PRPP (10 nmoles) at 37 ^{o}C for 0-120 min in a solution containing 45 mM Tris-HCl (pH 7.4), 1 mM EDTA, 10 mM $MgCl_2$ and dialysed hemolysate (15 mg protein) in a final volume

of 1.1 ml. Residual PRPP was measured after incubation for various time intervals by addition of [carboxyl-^{14}C]orotic acid and purified OPRT and ODC, and trapping the $^{14}CO_2$ released.

Acid phosphatase assay

Hydrolysis of p-nitrophenylphosphate at pH 7.4 and pH 5.0 was estimated by measuring the concentration of p-nitrophenol formed. Incubation mixtures contained 6 mM p-nitrophenylphosphate, 4 mM $MgCl_2$, 1.2 mM ouabain, hemolysate protein (4 mg) and 40 mM Tris-HCl (pH 7.4) or 40 mM sodium citrate (pH 5.0) in a final volume of 1.25 ml. After 10 min at 37 °C, 250 µl of ice-cold 30% trichloroacetic acid was added. Precipitated material was removed by centrifugation and 2 ml of 1 N NaOH was added. Absorption was measured at 410 nm using a Zeiss PM QII spectrophotometer. The extinction coefficient for p-nitrophenol was 17.6 x 10^3 M^{-1}. Correction was made for spontaneous hydrolysis of p-nitrophenylphosphate.

Purine nucleoside phosphorylase assay

The assay mixture contained 25 mM potassium phosphate (pH 7.4). 1 mM ethylene-diaminetetraacetic acid, 200 µM 8-[^{14}C]inosine and hemolysate protein (2-70 µg) in a total volume of 40 µl. After incubation for 20 min at 37 °C, the assay mixture was boiled for 2 min and centrifuged after addition of carrier hypoxanthine and inosine. Separation of product from substrate on PEI-cellulose plates and elution of radioactive compounds was achieved according to Reyes (1972). Aquasol was used as scintillation solution.

Protein

All enzyme activities are expressed in nmoles of product formed in 1 hr per mg of protein. Protein was determined according to Lowry et al. (1951).

Animals

The following species were used: Man - *Homo sapiens*, Swiss mouse - *Mus musculus*, Wistar rat - *Pattus norvegicus*, White New Zealand rabbit - *Oryctolagus cuniculus*, Beagle dog - *Canis familiaris*, Texel sheep - *Ovis aries*, Rhesus monkey - *Macaca mulatta*, Horse - *Equus caballus*, Pig - *Sus scrofa*, Cow - *Bos taurus*.

All enzyme activities were checked for linearity with regard to time and protein concentration. The linearity and validity of the PRPP assay has been described elsewhere (Tax & Veerkamp, 1977). Doubling the concentration of the substrates of PRPP synthetase did not result in an increase of enzyme activity with any of the hemolysates. PRPP synthetase activity from erythrocytes of several mammalian species was unstable to a freeze-thaw treatment. Therefore, this enzyme activity was always assayed in freshly prepared hemolysates obtained by hypotonic shock.

The results of our measurements of concentration and synthesis of PRPP in mammalian erythrocytes are given in Table 5.1. The PRPP concentration and

TABLE 5.1.

CONCENTRATION OF PRPP AND ACTIVITY OF PRPP SYNTHETASE, HGPRT, AND ACID PHOSPHATASE IN MAMMALIAN ERYTHROCYTES

Concentration of PRPP is given in nmoles/ml packed cells and enzyme activities in nmoles/hr per mg protein. The results represent the mean ± S D
Numbers in parentheses refer to the number of individuals.

Species	PRPP	PRPP synthetase	HGPRT	Acid phosphatase (pH 7 4)	Acid phosphatase (pH 5.0)
Man	1.52±0.21 (6)	45.0±2.7 (5)	95 5±8.3 (4)	15.7±4 5 (3)	49.2±2.8 (3)
Monkey	2.13±0.49 (4)	6.95±1.82 (4)	31.3±8.1 (10)	121 ±12 (3)	339 ±28 (3)
Dog	1.33±0.42 (4)	0.21±0.05 (4)	2.63±1.06 (4)	74.1±17 5 (4)	206 ±49 (4)
Mouse	11.7±0 9 (4)	20.1±1.2 (4)	9.59±0.90 (5)	274 ±44 (3)	496 ±27 (3)
Rat	10.3±2.4 (5)	25.3±5.4 (4)	3.24±0.36 (4)	198 ±35 (3)	383 ±50 (3)
Cattle	61.8±18.1 (7)	18.1±4.5 (4)	27.0±4.4 (5)	21.8±2.7 (3)	72.8±16.2 (4)
Rabbit	1.31±0.46 (5)	24.0±3.4 (4)	82.7±2.0 (4)	136 ±20 (3)	327 ±62 (3)
Pig	0.16±0.05 (4)	2.99±0.60 (4)	59.9±4.7 (4)	62.5±17.6 (4)	138 ±18 (4)
Sheep	0.87±0.13 (4)	11.1±4.1 (6)	53.0±10.2 (10)	33.5±5.8 (3)	88.5±14.6 (3)
Horse	62.4±7 8 (4)	5.88±0.40 (4)	0.59±0.11 (4)	40.5±16.5 (3)	121 ±34 (3)

PRPP synthetase activity in erythrocytes show large variations between the mammalian species, as was previously observed for the activities of purine phosphoribosyltransferases and orotate phosphoribosyltransferase (Tax et al., 1976). There seems to be no direct correlation between the PRPP concentration and the PRPP synthetase activity in the different species. The values for the PRPP concentration in human and pig erythrocytes are similar to those reported by Dean et al. (1973), but Micheli et al. (1975) reported concentrations of PRPP in erythrocytes of man, pig, cow and rat which were higher and showed a quite different mutual ratio.

PRPP stabilizes the activity of the APRT enzyme (Greene et al., 1970) and therefore a correlation might exist between PRPP concentration and APRT activity (Tax et al., 1976). No such correlation was found.

The data on HGPRT activity were also reported in our previous article (Tax et al., 1976) but are presented here in order to compare the synthetic capacity for PRPP with the capacity of a major PRPP utilizing enzyme. It has been generally believed that PRPP in erythrocytes is utilized entirely by phosphoribosyltransferase activities. HGPRT seems to be the most important consumer of PRPP in erythrocytes. OPRT activity is much lower than HGPRT activity in mammalian erythrocytes (Tax et al., 1976) while the substrate of APRT, adenine, is virtually absent in mammalian cells (Murray et al., 1971). Deficiency of HGPRT in man is associated with an increased erythrocyte PRPP concentration while deficiency of OPRT or APRT is not (Fox & Kelley, 1971). Steady state concentrations of PRPP cannot, of course, be deduced from capacities of synthesis and utilization measured at optimal conditions. Actual enzyme activities *in vivo* are modulated by the availability of substrates and the concentration of regulatory compounds, and will generally be much lower than maximal capacities. However, some correlations between HGPRT activity and PRPP concentration seem to hold. The increased concentration of PRPP in erythrocytes of patients with deficiency of HGPRT has already been mentioned. Partial deficiency of HGPRT in man is also associated with an increased concentration of PRPP in erythrocytes but the increase is less pronounced (Sperling et al., 1972; Tax & Veerkamp, 1977). HGPRT activity is nearly absent in horse erythrocytes and PRPP concentration in these cells is markedly high. Low activity of HGPRT and a relatively high concentration of PRPP is also found in erythrocytes of rat and mouse. In erythrocytes of all mammals except horse, rat and mouse the optimal activity of HGPRT appears to be much higher than the PRPP synthetase

capacity. At first sight, PRPP concentration of bovine erythrocytes is surprisingly high. In man, an increased PRPP concentration was observed in erythrocytes from patients with deficiency of purine nucleoside phosphorylase (Cohen et al., 1976, Tax & Veerkamp, 1977). This increase may be due to a decreased utilization of PRPP since the substrate of HGPRT, hypoxanthine, is not formed. Therefore we measured the activity of purine nucleoside phosphorylase in bovine erythrocytes. Enzyme activity was found to be very low (39 nmoles/hr per mg protein, 5 determinations) when compared with the activity in human erythrocytes (1308 nmoles/hr per mg protein). Duhm (1974) also reported a very low activity of purine nucleoside phosphorylase in bovine erythrocytes as well as an extremely low permeability for inosine.

Fox & Marchant (1974) described an alternative pathway for catabolism of PRPP which did not involve phosphoribosyltransferase activity. Alkaline phosphatase seemed to be the responsible enzyme. Hydrolysis of PRPP was found in extracts of all human tissues assayed except erythrocytes. We observed a substantial degradation of PRPP when this compound was heated in the presence of Mg^{2+} and human hemolysate. This destruction seemed to be an enzymatic process (Tax & Veerkamp, 1977). Incubation of PRPP at 37 ^{o}C and pH 7.4 with dialyzed hemolysate of man or rat in the presence of Mg^{2+} leads to destruction of PRPP(Table 5.2).Non-enzymatic hydrolysis of PRPP in the presence of Mg^{2+} does occur but is much slower than hydrolysis in the presence of hemolysate. Recovery of PRPP is nearly complete after incubation for 2 hr in the absence of Mg^{2+}. Human erythrocytes contain two acid phosphatases, one of which is strongly stimulated by Mg^{2+} (Kornfeld & Gregory, 1969). Therefore, acid phosphatase might be the enzyme responsible for destruction of PRPP. Commercial acid phosphatase from potato was found to hydrolyze PRPP at pH 7.4. Acid phosphatase activity was measured in hemolysates of all ten mammalian species, at both pH 7.4 and pH 5.0 with p-nitrophenylphosphate as substrate. $MgCl_2$ was included in the assay mixture. Ouabain was added to inhibit K^+-activated phosphatase activity associated with Na^+-K^+-ATPase (Heller & Hanahan, 1972). The results of these measurements are included in Table 5.1. Enzyme activity at pH 5.0 was much higher than at pH 7.4, the ratio being 2-3 for all species studied. Large variations in enzyme activity were observed between species. Acid phosphatase activity in human hemolysate is much lower than in rat hemolysate. Hydrolysis of PRPP by human hemolysate proceeds at a slower rate than hydrolysis by rat hemolysate (Table 5.2). This finding is therefore consistent with the

TABLE 5.2.

HYDROLYSIS OF PRPP BY DIALYZED HEMOLYSATE OF MAN AND RAT WITH AND WITHOUT Mg^{2+}

The amount of residual PRPP was measured after 0,60 and 120 min incubation at pH 7 4 and 37°C Values are the mean of two experiments

Hemolysate	Mg^{2+}	PRPP (nmoles)		
		0 min	60 min	120 min
-	+	9 8	8 2	6 9
Human	+	10 1	5 9	3 8
Human	-	9 7	8 9	8 3
Rat	+	9 7	3 3	0 2
Rat	-	10 4	9 3	8 9

hypothesis that the observed hydrolysis of PRPP is due to acid phosphatase activity. A similar enzymatic breakdown of PRPP was found with dialyzed hemolysates of mouse and rabbit.

The enzymatic hydrolysis of PRPP in mammalian hemolysates may interfere with *in vitro* investigations on PRPP metabolism when Mg^{2+} is present in the incubation mixture. The possible physiological significance of this catabolic pathway in intact erythrocytes requires further investigations.

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THE URINARY EXCRETION OF OROTIC ACID AND OROTIDINE, MEASURED BY AN ISOTOPE DILUTION ASSAY

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6.1. SUMMARY

Unknown concentrations of orotic acid can be measured by competition with a known amount of [carboxyl-^{14}C]orotic acid for reaction with a limiting amount of phosphoribosylpyrophosphate in the presence of orotate phosphoribosyltransferase and orotidine monophosphate decarboxylase. The dilution of the specific radioactivity in the product $^{14}CO_2$ is a sensitive and accurate measure of the amount of orotic acid present in the sample. Orotidine can also be determined after hydrolytic cleavage to orotic acid.

The method was used to measure orotic acid and orotidine in urine samples from newborns, healthy controls and patients with gout or deficiency of hypoxanthine-guanine phosphoribosyltransferase receiving allopurinol. Urinary excretion of orotic acid and orotidine in newborns was similar whether the infants were breast-fed or received milk powder. The excretion of orotidine was increased in all patients receiving allopurinol. After allopurinol administration orotic acid excretion was increased in gouty patients but close to normal values in patients with deficiency of hypoxanthine-guanine phosphoribosyltransferase. The results are discussed in relation to the mechanism by which allopurinol inhibits pyrimidine metabolism.

6.2. INTRODUCTION

Excessive urinary excretion of orotic acid is observed in hereditary orotic aciduria (1), after administration of allopurinol (2-4) or 6-azauridine (5),

in inherited disorders of the urea cycle (6, 7) and in deficiency of purine nucleoside phosphorylase (8). In some of these cases orotidine excretion is also increased. The colorimetric test (9) which is frequently used to quantitate orotic acid does not distinguish between orotic acid and orotidine (2) and several compounds interfere with this assay (10). Christopherson and Finch (11) described an isotope dilution assay for orotic acid which was specific and sensitive but rather time-consuming. Furthermore, the procedure included a column adsorption step with a low and variable recovery of orotic acid. In the present communication we describe a simple and rapid procedure for measuring orotic acid and orotidine. The assay is based on the competition between unlabeled orotic acid and [carboxyl-^{14}C]orotic acid for reaction with a limiting amount of phosphoribosylpyrophosphate (PRPP) in the presence of excess orotate phosphoribosyltransferase (OPRT, EC 2.4.2.10) and orotidine monophosphate decarboxylase (ODC, EC 4.1.1.23). The amount of unlabeled orotic acid can be calculated from the dilution of the specific activity of [carboxyl-^{14}C]orotic acid in the product CO_2 by reference to a standard curve.

The method was used to measure the urinary excretion of orotic acid and orotidine by healthy controls, by patients receiving allopurinol medication (patients with primary gout or deficiency of hypoxanthine-guanine phosphoribosyltransferase (HGPRT, EC 2.4.2.8)) and by newborns. Some of these newborns received dietary milk powder which was reported to contain a high concentration of orotic acid (12). The milk powder itself was also assayed for orotic acid and orotidine.

6.3. MATERIALS AND METHODS

Materials

PRPP was obtained from Boehringer, Mannheim. A preparation containing OPRT and ODC activities was obtained from either P-L Biochemicals or Sigma. [Carboxyl-^{14}C]orotic acid, [carboxyl-^{14}C]orotidine, omnifluor and aquasol were purchased from New England Nuclear Corp., Dreieichenhain, G.F.R., and [8-^{14}C]hypoxanthine from the Radiochemical Centre, Amersham. [8-^{14}C]-Xanthine was from ICN (Irvine, Ca.).

Urine specimens

Urine was collected for 24-h periods from 2 healthy children (age 10 and 15 y), 4 healthy adults, 3 patients with complete deficiency of HGPRT on allopurinol medication (200 mg/day), 1 patient with partial deficiency of HGPRT (300 mg of allopurinol per day) and 3 patients with primary gout receiving allopurinol (300 mg/day). Urine specimens were also obtained from 2 breast-fed newborns and 4 newborns receiving milk powder (full-term infants, age 4-9 days). The milk powder was Almiron M2 (Nutricia, the Netherlands).

Hydrolysis of orotidine

Orotidine was cleaved to orotic acid by heating 1-ml samples of urine with 0.2 ml of 12 M HCl at 100 ^{o}C for 2 h in sealed glass tubes.

Colorimetric procedure for orotic acid

Orotic acid was extracted from hydrolyzed or non-hydrolyzed samples with silicagel and measured colorimetrically according to Kesner et al. (10).

Isotope dilution procedure for orotic acid

3-ml samples of urine or 1-ml samples of hydrolyzed urine were applied to a Dowex-50 column (1 g dry weight, H^{+}-form). The column was eluted twice with 3 ml of distilled water. The eluate was neutralised with 2 M NaOH and brought to a final volume of 10 ml. The assay mixture (final volume 0.5 ml) contained 25-250 µl of eluate, 25 mM Tris/HCl (pH 7.4), 5 mM $MgCl_2$, 2 µM PRPP, 3 µM [carboxyl-^{14}C]orotic acid (41 mCi/mmol) and 0.1 mg or 0.3 mg OPRT/ODC mixture (enzyme preparations from P-L and Sigma, respectively). After incubation for 30 min at 37 ^{o}C, 0.2 ml of 1 M HCl was injected and shaking continued for 1 h. $^{14}CO_2$ was trapped and measured as described before (13). The radioactivity in $^{14}CO_2$ (F) is related to the amount of orotic acid in the sample (X) according to $F = A.B/(B+X)$, where A is the radioactivity in $^{14}CO_2$ measured in the absence of the unknown sample of orotic acid and B is the amount of [carboxyl-^{14}C]orotic acid. In calculating the concentration of orotidine, the results obtained with hydrolyzed samples were corrected for the amount of orotic acid present, as measured with non-hydrolyzed samples. Hydrolysis, purification procedure on Dowex and isotope dilution assay were all performed in duplicate.

Experiments with [^{14}C]labeled orotidine revealed that this compound was hydrolyzed completely to [^{14}C]orotic acid within 2 h in the presence of HCl at 100 ^{o}C. The orotic acid formed was stable under these conditions. Using the colorimetric procedure we then measured the sum of orotic acid and the orotic acid released from orotidine in hydrolyzed urine samples. When non-hydrolyzed urine was applied to the silicagel column, orotidine and orotic acid could not be separated completely. Above pH 1.0 orotidine was retained on the column, but the elution of orotic acid was not complete. At lower pH all of the orotic acid was eluted but also part of the orotidine. Since orotidine is also reactive with respect to the colorimetric test (2) we investigated whether the interference by orotidine was serious. Color yield per mol was found to be only 3.5 times higher with orotic acid than with orotidine. Since orotidine excretion is usually much higher than excretion of orotic acid, the colorimetric procedure apparently is not suitable to obtain separate values for orotic acid and orotidine.

To eliminate substances interfering with the isotope dilution assay, the urine samples had to be purified by running through a Dowex-50 H^{+} column. These substances could be hypoxanthine and xanthine, since these compounds interfered when added to the assay mixture. Using [^{14}C] labeled compounds it could be shown that these latter compounds are retained completely (> 98%) on the Dowex column, while all orotic acid is eluted (> 96%). The isotope dilution assay was not interfered by orotidine, uracil, uric acid or oxipurinol when these compounds were present in a concentration which was 20-fold higher than the concentration of orotic acid.

The radioactivity (F) present in trapped CO_2 was measured after addition of various known amounts of unlabeled orotic acid (Fig. 6.1). The values of F can also be calculated from the formula F = A.B/(B+X) when A and B are known (see Materials and Methods). The close agreement between the calculated and the experimental values of F (Fig.6.1) illustrates the accuracy of the method. It is of course essential for this method that other effects than isotope dilution do not contribute to the decrease of radioactivity in CO_2. This condition was tested in two ways. In one series of experiments a higher concentration of [carboxyl-^{14}C]orotic acid (B) was present in the assay mixture. The second test involved the addition of a known amount of unlabeled orotic acid to the column eluates. In both cases

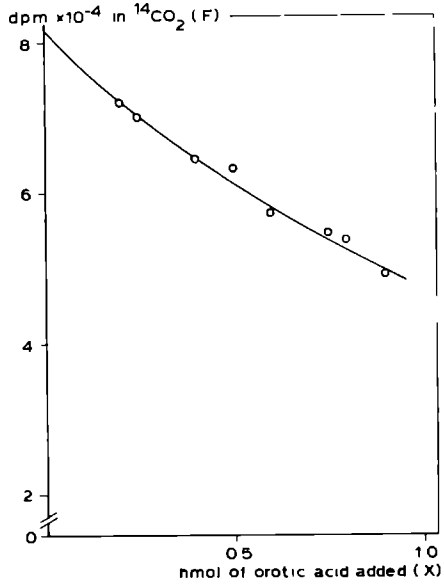

Fig. 6.1. Experimental and calculated values of radioactivity in $^{14}CO_2$ (F) as a function of the amount of orotic acid added (X). F was measured (open circles) by liquid scintillation counting of trapped $^{14}CO_2$ and calculated (drawn curve) according to F = A.B/(B+X) as described in Materials and Methods. Values for A and B averaged 82,000 dpm and 1.6 nmol, respectively.

consistent values of X were obtained for a given sample. This indicates that only isotope dilution is responsible for the decrease in radioactivity in CO_2.

The colorimetric test was used as a reference method for the isotope dilution assay. Two different samples of urine were hydrolyzed and applied to a Dowex column. The column eluates were assayed for orotic acid by both methods. Values obtained with the colorimetric test were 9.2 and 4.7 µg orotic acid per ml eluate. The corresponding values measured with the isotope dilution assay were 8.6 and 4.7 respectively. At low concentrations of orotic acid (< 1 µg per ml eluate) the colorimetric method is less precise due to its low sensitivity and agreement with the isotope dilution assay is worse.

The sensitivity of the isotope dilution assay is about 0.1 nmol (20 ng)

TABLE 6.1.

URINARY EXCRETION OF OROTIC ACID AND OROTIDINE IN NEWBORNS, CONTROLS AND PATIENTS RECEIVING ALLOPURINOL

	Creatinine	Orotic acid		Orotidine	
	mg/l	mg/l	mg/24 h	mg/l	mg/24 h
Newborns (n-6)	166 ± 56	0 7 ± 0 3	-	3 7 ± 1 3	-
Controls (n-6)	1204 ±248	1 3 ± 0 2	1 5 ± 0 5	4 5 ± 1 5	5 0 ± 0 9
Allopurinol-treated					
- Gout					
H v S.	910	7 6	11 2	39 7	58 6
J M.	862	25 2	33 3	107	142
J V	667	13 0	26 1	39 1	78 5
- Deficiency of HGPRT					
Ru W	379	4 4	J 7	86 8	72 6
Re W	424	2 6	2 2	123	104
P K	340	6 3	3 5	113	62 5
- Partial deficiency of HGPRT					
E F	809	1 4	2 1	73 5	112

Concentrations of orotic acid and orotidine in urine were measured by the isotope dilution procedure described in "Materials and Methods". For newborns and controls the means ± S D are given Complete sampling of 24-h urine could not be performed with newborns and therefore no values per 24 h are given for this group

of orotic acid. This procedure therefore allows the measurement of orotic acid and orotidine in urine samples without concentrating the urine. Values obtained for newborns, healthy controls and patients on allopurinol medication are given in Table 6.1. No difference in orotic acid and orotidine excretion was observed between children (10 and 15 y) and adults and therefore both groups were combined under the heading "controls". Orotidine excretion was increased in all patients receiving allopurinol, both in concentration and in amount per 24 h. Excretion of orotic acid after allopurinol administration was increased in gouty patients but was close to normal values in patients with partial or complete deficiency of HGPRT. Urinary concentrations of orotidine and orotic acid were similar in breast-fed newborns and in newborns receiving commercial milk powder. The newborns are therefore presented as one group in Table 6.1. The concentration of

orotic acid and orotidine in urine from newborns is slightly lower than in urine from adults, but when the excretion is calculated per mg creatinine the values are higher in urine from newborns (Table 6.1). The milk powder fed to some newborns contained no detectable orotidine and 15.8 $\pm$ 1.1 mg of orotic acid per 100 g powder (mean $\pm$ S.D. of six determinations).

6.5. DISCUSSION

The procedure described provides a specific, sensitive and relatively simple method for the measurement of orotic acid and orotidine in biological samples. The colorimetric assay described by Kesner et al. (10) does not distinguish between orotic acid and orotidine, and is less sensitive. The isotope dilution method published by Christopherson and Finch (11) involved an extraction procedure which yielded a low and variable recovery of orotic acid. Furthermore, the assay required thin-layer chromatography to separate the products from the substrate. No significant loss of orotic acid occurs in our method. The use of [carboxyl-^{14}C]orotic acid for the isotope dilution assay permits the isolation of the product (CO_2) during the assay. The interference of the assay by hypoxanthine indicates that some HGPRT activity may be present in the OPRT/ODC preparation (11). Urinary hypoxanthine, however, could be retained completely on the Dowex column. Orotidine could be measured after hydrolytic conversion to orotic acid. It appears to be difficult to separate orotidine from orotic acid. We obtained no complete separation with a silicagel column and even with high-pressure liquid chromatography the separation is poor (14).

Normal values for orotic acid and orotidine excretion obtained with this method are similar to values reported by other investigators (2-4, 15). Increased urinary excretion of orotidine after allopurinol administration was previously observed in normal subjects (2) and in patients with gout (3) or deficiency of HGPRT (4, 16). Normal subjects (4) and gouty patients (3) showed an increased orotic acid excretion after allopurinol administration. Both normal (4) and increased (16) excretion of orotic acid was reported for patients with deficiency of HGPRT receiving allopurinol. We found values close to normal for this group (Table 6.1). The orotidine excretion after allopurinol administration can be ascribed to inhibition of ODC by ribonucleotides of allopurinol and its oxidation product oxipurinol (16-18).

The increased excretion of orotic acid after administration of allopurinol may be due to inhibition of OPRT by accumulated orotidine monophosphate (18). A similar mechanism may be responsible for the increased excretion of orotidine and orotic acid following administration of 6-azauridine (5), 5-azacytidine (19)and 5-azaorotic acid (20). The absence of orotic aciduria after allopurinol administration in patients with deficiency of HGPRT is still consistent with this hypothesis, since the inhibition by orotidine monophosphate is competitive with PRPP (18) and PRPP concentration is increased in erythrocytes (13, 21) and fibroblasts (21) of these patients.

Urinary concentrations of orotic acid and orotidine were similar in newborns receiving milk powder and in breast-fed children. A high content of orotic acid (100-130 mg per 100 g of powder) was reported for commercial milk powder (12). We found a lower value (16 mg per 100 g powder). The difference may be caused by the use of milk powder from different manufacturers or may reflect the use of a more specific method (isotope dilution assay versus colorimetric determination). The dietary intake of orotic acid by newborns receiving milk powder (estimated to be about 10 mg per day) does not affect the urinary excretion of this compound. Diet apparently does not affect the urinary excretion of orotidine and orotic acid (15).

The isotope dilution method described is specific and very sensitive. Urinary concentrations of orotic acid as low as 1 µM can be quantitated without concentrating the sample. This method seems therefore also appropriate to investigate small disturbances of orotic acid metabolism due to drug-induced enzyme inhibition or secondary to deficiencies of purine nucleoside phosphorylase (8), of adenosine deaminase (22) or of enzymes of the urea cycle (6, 7).

6.6. REFERENCES

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CHAPTER 7

MECHANISM OF ALLOPURINOL-MEDIATED INHIBITION AND STABILIZATION OF HUMAN OROTATE PHOSPHORIBOSYLTRANSFERASE AND OROTIDINE PHOSPHATE DECARBOXYLASE

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7.1. SUMMARY

Allopurinol ribonucleotide and oxipurinol 7-ribonucleotide appeared to be strong inhibitors of orotidine phosphate decarboxylase in human hemolysates. The enzyme exhibited bimodal kinetics. The ribonucleotides of allopurinol and oxipurinol caused an inhibition of orotate phosphoribosyltransferase, which appeared to be due to accumulation of OMP. Inhibition of OMP was competitive with respect to phosphoribosylpyrophosphate with a K_1 value of 11 µM. The inhibition of ODC and OPRT activity may cause the increased urinary excretion of orotidine and orotic acid, respectively, observed after allopurinol therapy. Values measured for OPRT activity in intact erythrocytes and in hemolysates agreed very well. Therefore OPRT activity does not decrease during cell lysis and extraction. Hypoxanthine-guanine phosphoribosyltransferase deficiency as well as allopurinol therapy led to a marked increase in OPRT and ODC activities in human hemolysates. In lysates from leukocytes only a slight increase of ODC activity was observed, while OPRT activity did not differ significantly from the controls. *In vitro* incubations of hemolysates demonstrated a considerable increase of the stability of OPRT by addition of OMP or PRPP and of ODC by addition of OMP, PRPP, UMP and the ribonucleotides of allopurinol and oxipurinol. These findings suggest that the apparent increase of OPRT and ODC activity after allopurinol therapy is due to stabilization of the enzymes during the life span of the erythrocytes.

7.2. INTRODUCTION

Allopurinol inhibits the final enzyme of purine metabolism in man, xanthine

oxidase (EC 1.2.3.2.) and is an effective agent for treatment of hyperuricemia (1). Allopurinol therapy also interferes with pyrimidine metabolism as indicated by an increased excretion of orotidine and orotic acid in urine (2, 3). Subsequent investigations have established that allopurinol and its major metabolite oxipurinol inhibit pyrimidine biosynthesis in cultured human cells by interfering with the conversion of orotic acid to UMP (4, 5). The increased excretion of orotidine has been ascribed to inhibition of orotidine 5'-monophosphate decarboxylase (EC 4.1.1.23) by ribonucleotides of allopurinol and oxipurinol. The increase in excretion of orotic acid suggests that inhibition of orotate phosphoribosyltransferase (EC 2.4.2.10) may also occur but the exact mechanism has not yet been elucidated.

In addition it was observed that activities of OPRT and ODC in erythrocytes from patients receiving allopurinol were markedly elevated. This phenomenon has been attributed to enzyme stabilization *in vivo* (7) or enzyme activation (8). The apparent increase in activity might also be due to stabilization of the enzymes during cell lysis and extraction rather than stabilization *in vitro* (5, 9).

In the present study we have examined the mechanisms responsible for enzyme inhibition on the one hand and apparently increased enzyme activities on the other hand. In these studies we used both normal blood cells and cells obtained from patients deficient in hypoxanthine-guanine phosphoribosyltransferase (EC 2.4.2.8) activity. Our results suggest that allopurinol-induced inhibition of ODC activity is associated with inhibition of OPRT activity by accumulated OMP. In addition, evidence is presented that the apparent increase in OPRT and ODC activities is probably due to stabilization of the enzymes during the life span of the erythrocyte.

7.3. MATERIALS AND METHODS

Materials

Phosphoribosylpyrophosphate tetrasodium salt was purchased from Boehringer, Mannheim. Orotic acid monosodium salt and orotidine 5'-monophosphoric acid trisodium salt were obtained from Sigma Chemical Co., St.Louis, Mo. (U.S.A.). Allopurinol, oxipurinol and allopurinol ribonucleotide were gifts from Dr. G.B.Elion, Burroughs-Wellcome Co., Research Triangle Park, N.C. (U.S.A.).

Oxipurinol 7-ribonucleotide was generously provided by Henning Chemie & Pharmawerk, Berlin, West Germany. Dextran T 500 and Sephadex G-25 (coarse) were obtained from Pharmacia, Uppsala, Sweden. [Carboxyl-^{14}C]orotic acid (42.4 mCi/m-mole), [carboxyl-^{14}C] orotidine 5'-monophosphate (36.9 mCi/m-mole) and [6-^{14}C]orotic acid (49.1 mCi/m-mole) were obtained from New England Nuclear Corp., Dreieichenhain, West Germany, as well as omnifluor and aquasol. Aluminum sheets (20 x 20 cm) precoated with 0.1 mm of polyethyleneimine-cellulose were purchased from Merck, Darmstadt, West Germany. The other chemicals were of the highest quality commercially available.

Blood samples

Venous blood samples were obtained from three gouty patients receiving allopurinol (300 mg/day) for at least 6 months, from three children with HGPRT-deficiency who have been on allopurinol medication (200 mg/day) for several years and from a gouty adult (E.F.) with partial deficiency of HGPRT (1 per cent of normal value) who also has received the drug (300 mg per day) for several years. Two children with HGPRT-deficiency exhibited all symptoms of the Lesch-Nyhan syndrome including automutilation (10) while the third one (P.K.) had no severe neurological lesions. Adult volunteers served as control group.

Preparation of cells and cell extracts

In order to separate leukocytes from erythrocytes by differential sedimentation, dextran was added to the blood samples (11). After the erythrocytes had settled, they were washed twice with Tris-buffered saline (pH 7.4). The cells were used intact or lysed by rapidly freezing and thawing twice. Leukocytes were centrifuged at 1500 rpm and remaining erythrocytes were removed by a hypotonic shock for 60 sec after which isotonicity was restored. Leukocytes were washed with phosphate-buffered saline and lysed either by prolonged hypotonic shock or by sonication. Lysates of leukocytes were immediately assayed for enzyme activities while hemolysates could be stored at -20^{o} for several weeks without detectable loss of OPRT and ODC activity.

Analytical procedures

The actual PRPP content of stock solutions was determined enzymatically using [8-^{14}C]adenine and adenine phosphoribosyltransferase partially purified

from human erythrocytes (12) The reaction was stopped by boiling for 2 min and AMP was separated from adenine by thin-layer chromatography on PEI-cellulose (13). Enzyme activities were estimated with radiochemical methods using ^{14}C-labeled compounds. Determination of radioactivity was performed in a Packard 3380 liquid-scintillation spectrophotometer with external standardization. Protein was determined according to Lowry et al. (14). Specific enzyme activities are expressed in nmoles of product formed in 1 hr per mg of protein at 37° under the assay conditions specified below. All enzyme assays were checked on linearity with respect to enzyme concentration and time.

Assay of orotate phosphoribosyltransferase. Assay I

Incubation mixtures contained 0.05 M Tris-HCl buffer (pH 7.4), 5 mM $MgCl_2$, 0.7 mM PRPP, 0.3 mM [carboxyl-^{14}C]orotic acid (0.15 mCi/m-mole) and enzyme protein in a total volume of 0.6 ml. Reactions were carried out in scintillation vials sealed with rubber caps. The $^{14}CO_2$ evolved was trapped in 0.2 ml of a mixture of ethyleneglycol-ethanolamine (2 1, v/v) present in a small polypropylene tube fitted in a slightly larger tube. After incubation for 10-60 min with shaking, OPRT activity was stopped by injection of 0.2 ml 0.25 M neutralized EDTA. Because ODC does not require Mg^{2+}-ions, decarboxylation can be allowed to proceed for another hour. Injection of 0.2 ml 5 M perchloric acid removed all $^{14}CO_2$ from the reaction mixture within 1 hr. The small tube containing trapped $^{14}CO_2$ was transferred to a scintillation vial with 10 ml of toluene-methanol (2 1, v/v) containing 4 g omnifluor per litre. Blanks contained no enzyme protein.

This assay was also employed when OPRT activity was determined in intact erythrocytes. A 25% suspension of erythrocytes in phosphate-buffered saline containing 0.1% glucose and 0.1% $MgCl_2$ was preincubated for 1 hr resulting in intracellular PRPP synthesis. [Carboxyl-^{14}C]orotic acid was added to 500 µl of this suspension to reach a final concentration of 0.3 mM (sp. act. 0.15 mCi/m-mole). During a 30-min incubation $^{14}CO_2$ production was trapped as described above. Reaction was terminated by injection of perchloric acid. Preliminary experiments had shown that intracellular orotic acid concentration almost instantaneously equals the medium concentration.

Assay II

In this assay unreacted orotate is separated from the products which are

synthesized from orotate by OPRT and any consecutive enzyme which may be present (15). The reaction mixture contained 40 mM Tris-HCl (pH 7.4), 0.1 mM [6-^{14}C]orotic acid (49.1 mCi/m-mole), 5 mM $MgCl_2$, 1.1 mM PRPP and enzyme protein in a final volume of 50 µl. The mixture was incubated for 30 min in small polypropylene tubes. Reaction was terminated by immersing the tubes in boiling water for 2 min. Carrier orotic acid and uridine were added and precipitated protein removed by centrifugation for 10 min at 4500 g in a Misco centrifuge (Microchemical Specialities Co., Berkeley, California, U.S.A.). Ten-µl samples of the supernatant were spotted on PEI-cellulose thin-layer plates. Development was accomplished with 0.2 M LiCl (saturated with boric acid and adjusted to pH 4.5)-ethanol (1:1, v/v). Chromatograms were dried at room temperature. The spots were visualized under u.v. light and cut out. In order to circumvent any self absorption of radioactivity, compounds were eluted prior to counting by shaking with 1.0 ml of 0.1 M HCl-0.2 M KCl for 40 min in scintillation vials. Ten ml of aquasol was added and radioactivity measured. Blanks were obtained by immersing the tubes in boiling water for 2 min prior to incubation. The conversion of substrate into products was calculated by comparing net radioactivity in products derived from orotate with the total amount of radioactivity present. A higher PRPP concentration than in assay I was necessary since the higher protein concentration employed seemed to be associated with an increase of enzymatic breakdown of PRPP.

Assay of orotidine-5'-phosphate decarboxylase

Cell lysates, appropriately diluted with 50 mM Tris-HCl buffer (pH 7.4) were incubated with 0.1 mM [carboxyl-^{14}C]orotidine 5'-monophosphate (0.2 mCi/m-mole) for 10-60 min in a final volume of 0.55 ml. Reaction was terminated by injection of 0.2 ml 5 M perchloric acid. Production of $^{14}CO_2$ was measured as described for OPRT, assay I.

Stabilization studies

Hemolyzed red cells were diluted with 9 vol of Tris-HCl buffer (50 mM, pH 7.4) and incubated for 16 hr at 37°. To avoid bacterial contamination penicillin-G (100 U/ml) and streptomycin (100 µg/ml) were present in the incubation vessels. When it was necessary to remove inhibitors of OPRT or ODC before enzyme assay lysates were passed through Sephadex G-25 columns

after incubation. 10 mM Tris-HCl (pH 7.4) was used for suspending the Sephadex powder as well as for eluting the protein from the column. The enzymes were eluted in the void volume together with hemoglobin.

7.4. RESULTS

Activities of orotate phosphoribosyltransferase and orotidine 5'-phosphate decarboxylase in circulating blood cells

OPRT and ODC activities were elevated in hemolysates from the allopurinol-treated group when compared with the control group (Table 7.1).This increase in enzyme activity was seen with both the HGPRT-deficient patients and the gouty patients. Enzyme activities were also measured in HGPRT-deficient subject P.K. before allopurinol therapy was started. Values averaged 1.0 nmole/hr per mg protein for OPRT, and 1.7 nmole/hr per mg protein for ODC activity.

In lysed leukocytes OPRT activity of the allopurinol-treated patients was not higher than in controls. ODC activity was slightly higher than in 6 control subjects.

OPRT activity was also measured in intact erythrocytes after preincubation in a PRPP-generating system. For control subjects, OPRT activities in intact erythrocytes and in lysed erythrocytes agreed very well (Table 7.2). However, OPRT assay in intact erythrocytes from allopurinol-treated patients resulted always in lower values when compared with corresponding hemolysates.

Effect of allopurinol and its metabolites on activity of orotidine 5'-phosphate decarboxylase

Allopurinol and oxipurinol were found to have no effect on ODC activity in hemolysates. However, when a hemolysate was preincubated with allopurinol or oxipurinol (1 mM) in the presence of 1 mM PRPP compounds were synthesized which strongly inhibited ODC activity. Longer periods of preincubation resulted in stronger inhibition of ODC. The inhibitors formed during the preincubation period were shown to be competitive with respect to orotidine 5'-monophosphate (Fig.7.1).The inhibitory agents are presumably allopurinol ribonucleotide and oxipurinol 7-ribonucleotide. These compounds appeared to be competitive inhibitors of ODC (Fig.7.2).Oxipurinol 7-ribonucleotide

TABLE 7.1.

OROTATE PHOSPHORIBOSYLTRANSFERASE AND OROTIDINE MONOPHOSPHATE DECARBOXYLASE ACTIVITIES IN HUMAN LEUKOCYTES AND ERYTHROCYTES

	Leukocytes		Erythrocytes	
Subject	OPRT	ODC	OPRT	ODC
Control group (n=6)	1.65 ± 0.53*	5.09 ± .12	0.19 ± 0.05	0.34 ± 0.10
Allopurinol treated HGPRT-deficient				
L.F.	0.88	6.70	0.72	1.14
P.K.	1.28	8.39	1.23	2.42
Re.W.	1.00	7.75	1.09	2.94
Ru.W.	.39	7.04	1.87	2.76
Gouty				
E.v.d.W.	2.06	6.55	0.51	0.70
H.v.S.	1.26	5.34	0.54	1.60
H.V.	1.08	6.30	0.31	0.57

* Enzyme activity ± SD in nmoles/hr per mg protein

TABLE 7.2.

OROTATE PHOSPHORIBOSYLTRANSFERASE ACTIVITY IN INTACT AND LYSED HUMAN ERYTHROCYTES

Subject	Intact cells	Hemolysate
Controls:		
G.H.	0.22*	0.19
W.T.	0.22	0.22
W.G.	0.14	0.12
Allopurinol treated·		
H. v.S.	0.59	1.09
P.K.	0.47	1.23
Re. W.	0.81	1.49
Ru. W.	0.84	1.87

* Enzyme activity was measured by assay I and is given in nmoles/hr per mg protein.

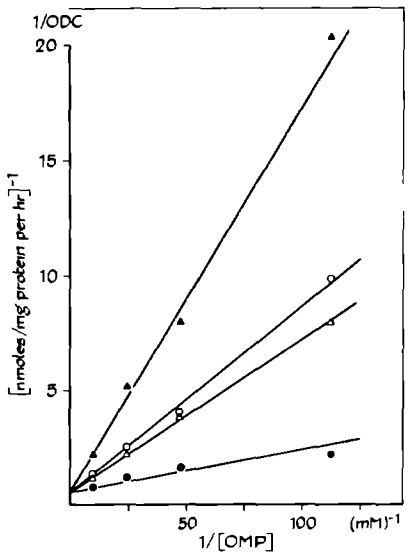

Fig. 7.1. Inhibition of ODC in hemolysate after incubation with allopurinol or oxipurinol in the presence of 1 mM PRPP. Control (●), allopurinol during 30 min (○), oxipurinol during 30 min (△), oxipurinol during 60 min (▲).

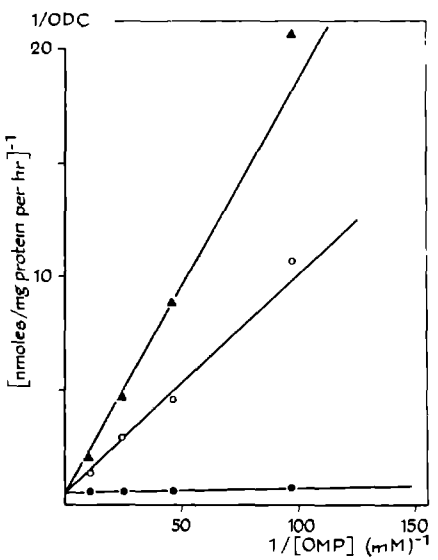

Fig. 7.2. Inhibition of ODC in hemolysate by ribonucleotides of allopurinol and oxipurinol. Control (●), 1.1 x 10^{-5} M oxipurinol 7-ribonucleotide (▲), 5 x 10^{-4} M allopurinol ribonucleotide (○).

(K_i value 0.06 μM) was a much stronger inhibitor of ODC than the former compound (K_i value 5 μM).

The synthesis of allopurinol 1-ribonucleotide is catalyzed by HGPRT, since no inhibition of ODC was observed when allopurinol and PRPP were incubated with HGPRT-deficient lysate. HGPRT activity did not appear necessary for an inhibitory oxipurinol ribonucleotide to be formed (Table 7.3). Xanthosine monophosphate was reported to be a competitive inhibitor of ODC activity (3, 6). It was also inhibitory in our assay system. However, preincubation of xanthine and PRPP with hemolysate did not result in a detectable inhibition of the ODC activity.

We found bimodal kinetics for the ODC activity in hemolysates. At (unphysiologically) high concentrations of substrate the ODC enzyme shows higher apparent values for both K_m and V_{max} (Fig. 7.3). At OMP concentrations lower than about 30 μM a K_m value of 1.7 $\pm$ 0.4 μM (mean $\pm$ S.D., 4 determinations) was found. Values for high K_m averaged 33 μM. Similar kinetics were observed when the enzyme concentration was 4-fold higher. This finding of bimodal kinetics does not affect the K_i values reported above for oxipurinol 7-ribonucleotide and allopurinol ribonucleotide. These values were determined at the low concentration range of OMP.

Effect of allopurinol and its metabolites on activity of orotate phosphoribosyltransferase

Unless otherwise indicated all results described in this section have been obtained using assay II for OPRT assay. Allopurinol has no inhibitory effect on OPRT activity at saturating concentrations of orotic acid and PRPP. Even at concentrations of PRPP which are suboptimal for OPRT we found no inhibition of OPRT activity by allopurinol. Under these conditions hypoxanthine strongly inhibits OPRT activity (Table 7.4). This inhibition is probably due to consumption of PRPP in a HGPRT-catalyzed reaction since no inhibition was found with HGPRT-deficient lysate. Inhibition is nearly absent when PRPP concentration is high.

A small but significant inhibition of OPRT activity was found when allopurinol 1-ribonucleotide or oxipurinol 7-ribonucleotide was present in the assay mixture. Inhibition was stronger at lower PRPP concentrations and also increased with longer incubation periods. This type of inhibition could be explained by assuming that the presence of ribonucleotides of allo-

TABLE 7.3.

RELATIVE OROTIDINE MONOPHOSPHATE DECARBOXYLASE ACTIVITY IN HEMOLYSATES AFTER PREINCUBATION WITH ALLOPURINOL AND OXIPURINOL

Preincubation conditions	Normal	HGPRT-deficient
PRPP	100*	100*
Allopurinol + PRPP	42	100
Oxipurinol + PRPP	42	12

* 100% - value was 0.7 nmoles/hr per mg protein

* 100% - value was 2.8 nmoles/hr per mg protein

Time of preincubation was 60 min, compounds were added in a concentration of 1 mM OMP concentration during assay was 0.1 mM.

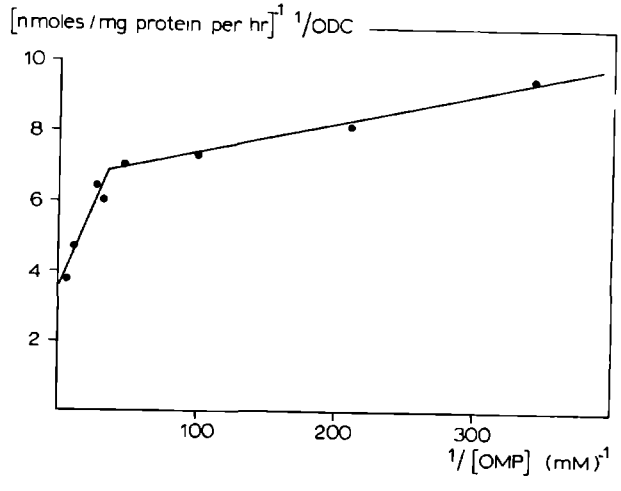

Fig. 7.3.

Biphasic kinetics of ODC in human hemolysate.

TABLE 7.4.

RELATIVE OROTATE PHOSPHORIBOSYLTRANSFERASE ACTIVITY IN HEMOLYSATES IN THE PRESENCE OF ALLOPURINOL AND HYPOXANTHINE

Addition \ PRPP (mM)	Normal		HGPRT-deficient	
	0.5	2.0	0.5	2.0
Hypoxanthine (1 mM)	39	82	103	104
Allopurinol (1 mM)	107	94	112	102

Control values (set at 100%) were 0.19, 0.20, 1.1 and 1.2 nmoles/hr per mg protein, respectively. Results are the mean of two experiments.

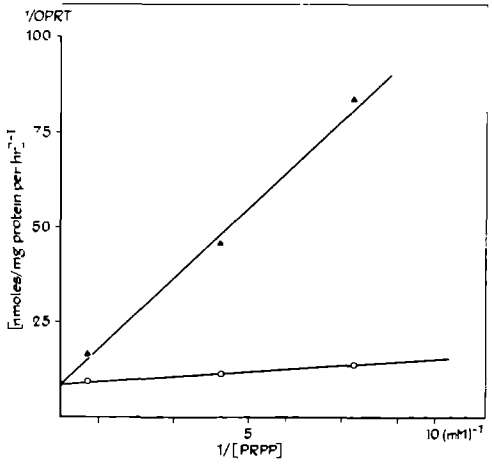

Fig. 7.4.
Inhibition of OPRT in hemolysate by OMP. Control (○), 2 x 10^{-4} M OMP (▲).

purinol and oxipurinol causes an accumulation of an inhibitor. Because ODC activity is inhibited by ribonucleotides of allopurinol and oxipurinol (see above) OMP might accumulate during their presence. This compound was therefore studied with regard to possible inhibition of OPRT activity.

When studying the effect of OMP on OPRT, care must be taken to prevent

utilization of this compound. Some dephosphorylation of OMP was found to occur in hemolysates but this reaction was of minor importance at the low concentrations of OMP used in these studies. Decarboxylation of OMP is much more of a problem since ODC activity in hemolysates is twice as high as OPRT activity. In order to prevent a decrease in the OMP concentration during the OPRT assay due to ODC activity, allopurinol ribonucleotide (1 mM) was added to all reaction mixtures (including controls) when studying the effect of OMP. Under these conditions OMP appeared to be a potent inhibitor of OPRT activity. Inhibition is competitive with respect to PRPP (Fig.7.4). When the K_i value is calculated from the Lineweaver-Burk plots, a value of 13 μM is obtained. However, this value depends on the PRPP concentration. Because of the known instability of PRPP (16) the values of the PRPP concentrations may be to some extent inaccurate, which affects the calculated value of K_i. Therefore the K_i value for OMP was also determined according to the Dixon method (17), using various concentrations of inhibitor and two different concentrations of PRPP. This method yielded a K_i value of 11 μM (Fig.7.5).

Several nucleotides appeared to be inhibitors of OPRT (Table 7.5). Assay I for OPRT was used in these experiments. Prior to testing the effect of these nucleotides on OPRT activity, their non-interference with the ODC reaction was ascertained. Inhibition was competitive with respect to PRPP for all inhibitors tested, except TTP. This compound strongly inhibits OPRT activity even at a high concentration of PRPP.

Stabilization studies

When hemolysates were diluted with Tris-HCl buffer (pH 7.4) and incubated for 16 hr at 37°, both OPRT and ODC activity decreased strongly. The extent of this decrease was very different for various hemolysates (Table 7.6). This great variability is reflected in the variability of the values found after incubation in the presence of stabilizing compounds, since these values were calculated by comparison with their respective control values. The presence of 0.1 mM allopurinol ribonucleotide or oxipurinol 7-ribonucleotide appeared to stabilize ODC activity, but no effect on OPRT activity was observed. Allopurinol ribonucleotide had similar effects at 1 mM, but no stabilization could be measured with 1 mM oxipurinol 7-ribonucleotide. As a matter of fact, OPRT and ODC activities were under these conditions even lower than in controls without any addition. Stabilization of ODC

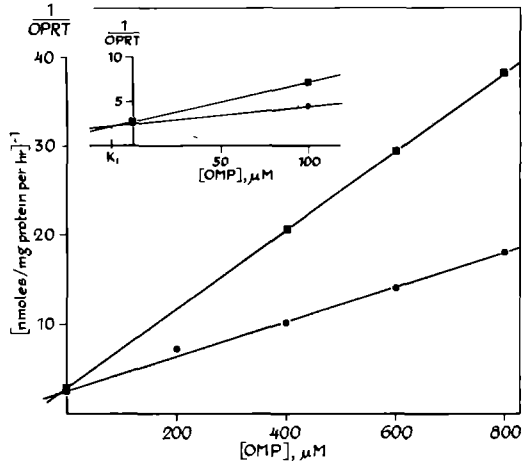

Fig. 7.5.

Determination of K_i value of OMP for OPRT from hemolysate according to Dixon (17). OPRT activity was measured with assay II (see Methods). Allopurinol ribonucleotide (1 mM) was present to prevent decarboxylation of OMP. Concentration of PRPP was 1 mM (●) or 0.5 mM (■).

TABLE 7.5.

EFFECT OF NUCLEOTIDES ON OROTATE PHOSPHORIBOSYLTRANSFERASE ACTIVITY IN HEMOLYSATE

Addition	Relative OPRT activity
None	100
TTP	14 ± 1
AMP	48 ± 2
GMP	54 ± 1
ATP	62 ± 1
IMP	71 ± 2
TMP	75 ± 2
ADP	76 ± 1
CMP	89 ± 4
c-AMP	100 ± 5

Mean and deviation from the mean of two experiments are given as percents of the control. Concentration of nucleotides was 5 mM, and concentration of PRPP was 0.2 mM.

TABLE 7.6.

RELATIVE ACTIVITY OF OPRT AND ODC AFTER OVERNIGHT INCUBATION AT 37°C IN THE PRESENCE OF NUCLEOTIDES

Addition	Nr of expts.	OPRT	ODC
None		100	100
UMP (1 mM)	2	115 (112-118)	236 (219-253)
OMP (0.1 mM)	2	119 (113-124)	204 (181-226)
OMP (1 mM)	6	182 (157-259)	508 (208-1033)
Oxipurino. ribo-ucleotide (0.1 mM)	3	92 (80-100)	258 (179-348)
Allopurinol ribo-ucleotide (0.1 mM)	3	94 (87-130)	305 (187-528)
Allopurinol ribonucleotide (1 mM)	3	91 (72-107)	318 (273-404)

Values between brackets indicate the range observed with different hemolysates. Before incubation relative OPRT activity was 1233 (672-2667, n=10) and relative ODC activity was 623 (396-1255, n=10)

occurred also when UMP was present at a concentration of 1 mM, but was not detectable at 0.1 mM. Again, no stabilizing effect on OPRT was observed. OPRT activity, as well as ODC activity was stabilized by OMP at 1 mM. Stability of both enzymes was not significantly influenced by any of the following compounds at 1 mM concentration: orotic acid, allopurinol, oxipurinol, TMP or TTP. OPRT and ODC activities in hemolysates incubated for 5 hr in Tris-HCl buffer were only 6 and 30 per cent, respectively (mean of 2 experiments) of the enzyme activities before incubation. When the incubation was performed in phosphate buffer (50 mM, pH 7.4) instead of Tris-HCl buffer, both OPRT and ODC activity appeared much more stable. Activities were 95 and 81 per cent of the starting value, respectively (mean of 2 experiments). Complete stabilization of OPRT and ODC activities (110 and 96 per cent respectively of the starting value, 2 experiments) was observed in Tris-HCl buffer during the 5-hr period when at 30 min intervals PRPP was added to a final concentration of 0.4 mM.

7.5. DISCUSSION

The effects of allopurinol on OPRT and ODC activities include enzyme inhibition on one hand leading to increased excretion of orotic acid and orotidine (2, 3) and an apparent increase of enzyme activities in erythrocytes on the other hand (7,8)(Table 7.1). The observed increase in orotidine excretion was ascribed to inhibition of the ODC enzyme (3, 7). Our experiments sustain this explanation. Allopurinol ribonucleotide and xanthosine 5'-monophosphate are potent inhibitors of ODC activity (3, 4). We found no inhibition of ODC after preincubation of hemolysates with xanthine and PRPP, despite the fact that XMP inhibited ODC activity. However, xanthine is a much poorer substrate for HGPRT than is allopurinol (18). Allopurinol ribonucleotide is readily formed from allopurinol and PRPP, and strongly inhibits ODC activity (Fig. 7.1, 7.2). Since HGPRT activity is necessary for the formation of this inhibitor (Table 7.3) allopurinol-mediated inhibition of ODC activity cannot be ascribed to allopurinol ribonucleotide in HGPRT-deficient patients. Several studies (5-7) have stressed the importance of oxipurinol metabolites as inhibitors of ODC. Oxipurinol is converted to an inhibitor of ODC activity by HGPRT-deficient hemolysate in the presence of PRPP (Table 7.3). Oxipurinol 7-ribonucleotide may be responsible for inhibition of ODC in HGPRT-deficient patients treated with allopurinol (6). Oxipurinol ribonucleotides may be more important *in vivo* with regard to ODC inhibition than is allopurinol ribonucleotide. This is suggested by comparison of their concentrations in rat liver after allopurinol administration (19) and of their K_i values. The K_i value of oxipurinol 7-ribonucleotide for the ODC enzyme of human hemolysate (0.06 µM) is similar to the values reported for yeast and rat liver (6). For allopurinol ribonucleotide the observed K_i value was slightly higher than the values for the enzyme from erythrocytes (3), yeast and rat liver (6).

Several nucleotides inhibit OPRT activity (Table 7.5) but have no effect on ODC activity of human hemolysates. CMP, GMP and AMP were reported to inhibit cow brain ODC activity (20) while GMP, AMP and IMP did not affect ODC activity in human hemolysates (3). K_i values for GMP, AMP and IMP were all determined to be greater than 100 µM with the enzyme from yeast (6). UMP inhibition of rat liver ODC has been reported (21) but this was not confirmed by other investigators (22).

The ODC enzyme from rat liver (6), yeast (6) and human fibroblasts (23) exhibits bimodal kinetics, as in human hemolysate (Fig. 7.3). The K_m value

at low OMP concentrations (1.7 μM) is close to the values reported for erythrocytes (3), rat liver (6), fibroblasts (23) and yeast (6). The K_m value at high concentrations of OMP (33 μM) was much higher than in rat liver (6) and yeast (6). Very recently a triphasic Lineweaver-Burk plot was found with partially purified ODC from human erythrocytes (24).

Competitive inhibition of OPRT by allopurinol has been suggested (2) as an explanation for the observed increase in excretion of orotic acid during allopurinol therapy, but our results do not support this hypothesis. Since OMP might accumulate during allopurinol therapy by inhibition of ODC activity, the competitive inhibition of OPRT activity by OMP may be a probable cause for the excretion of orotic acid. Inhibition by OMP of OPRT activity in baker's yeast was previously reported (25, 26) but the mechanism of inhibition was not clarified. Recently a similar competitive inhibition was also demonstrated in rat liver (27). Because tissue concentrations of PRPP are probably much below the K_m value of PRPP for the OPRT enzyme (28, 29), competitive inhibition of OPRT by OMP may be of physiological significance.

Several other nucleotides inhibit OPRT activity in hemolysates (Table 7.5) and in other systems. UMP inhibition of OPRT was found with rat liver (27) but not with the enzyme from rat hepatoma cells (30) and baker's yeast (26). The enzyme from yeast was inhibited by CMP and GMP, but not by adenine nucleotides or uridine nucleotides (26). IMP, XMP and GMP inhibited only slightly the activity of a pyrimidine phosphoribosyltransferase from murine leukemia cells while CMP, TMP and AMP were ineffective (31).

OPRT activity may also be inhibited by depletion of PRPP. A decrease of PRPP concentration has been reported in human red cells following a single dose of allopurinol (32). No such decrease was found in human fibroblasts (4) when allopurinol concentration was 0.1 mM which is much higher than the plasma levels in man (33). Allopurinol had no detectable effect under conditions at which hypoxanthine caused a marked inhibition of OPRT by PRPP depletion (Table 7.4). This is consistent with the high K_m value of allopurinol (1 mM) for HGPRT when compared with the natural substrate hypoxanthine (2.4 μM) (18). Since allopurinol has also a short halflive (34) depletion of PRPP does not appear to be the mechanism responsible for allopurinol-induced orotic aciduria.

The apparent increase of erythrocyte OPRT and ODC activities after allopurinol therapy (Table 7.1) has been attributed to stabilization *in vivo*

(7), to enzyme activation (8), and most recently to stabilization of these enzymes during cell lysis and extraction (5, 9). Our results do not support this last suggestion. Measurement of OPRT activity in intact and lysed erythrocytes of controls reveals that these activities agree very well (Table 7.2). In intact erythrocytes from allopurinol-treated patients the production of $^{14}CO_2$ from [carboxyl-^{14}C]orotic acid is consistently lower than in lysates. This can be attributed to inhibition of ODC activity by ribonucleotides of allopurinol and/or oxipurinol. Assay II was not appropriate for measuring OPRT activity in intact erythrocytes, because of technical complications. Besides, this assay is also sensitive to indirect inhibition by OMP due to the presence of inhibitors of ODC.

Decrease of ODC activity during incubation at 37° is partially prevented by OMP and also by allopurinol ribonucleotide, UMP and oxipurinol 7-ribonucleotide (Table 7.6). Because these latter compounds compete with OMP at the ODC enzyme, the stabilizing effect might be called pseudosubstrate stabilization. A similar *in vitro* stabilization of ODC has been found with the enzyme purified from yeast (6). An increased thermal stability of ODC activity was present in lymphoblasts after incubation with oxipurinol (5) and in rat liver after administration of allopurinol (35). An increased heat stability of ODC from cow brain was found in the presence of competitive inhibitors of the enzyme (20). The occurrence of biphasic kinetics for the ODC enzyme from hemolysate (Fig. 7.3) might be associated with a change in quaternary structure brought about by OMP (31). All findings suggest that both OMP and inhibitors of ODC which are competitive with OMP may change the quaternary structure of the ODC enzyme and thereby affect the stability and activity of the enzyme. The inhibition of the enzyme activities observed after incubation with 1 mM oxipurinol ribonucleotide can be attributed to the tight binding of this compound to the enzyme protein during the gel filtration. The same phenomenon was found with lymphoblast extracts (5).

Stabilization of OPRT was observed in the presence of OMP which appeared to be a competitive inhibitor of OPRT with respect to PRPP. TTP, an inhibitor of OPRT which is not competitive with regard to PRPP, has no stabilizing effect. PRPP itself gives complete protection of both OPRT and ODC activities when loss of this instable compound is compensated for by addition of fresh PRPP at regular intervals. The stabilizing effect of phosphate on OPRT and ODC activities is probably mediated by PRPP since phosphate is an activator of PRPP synthesis in hemolysates (36). Stabilization by PRPP could account

for the apparent increase of both OPRT and ODC activities measured in the HGPRT-deficient subject P.K. before allopurinol therapy was started. Erythrocyte PRPP concentrations are elevated in HGPRT-deficient patients (37). A similar increase of OPRT and ODC activities was reported previously (38) in HGPRT-deficient patients without allopurinol therapy. It was suggested from thermal inactivation studies that the increase was not due to stabilization by PRPP. Because of the instability of PRPP these studies do not seem very suitable to investigate a possible stabilization by PRPP.

The ODC activity in leukocytes (Table 7.1) is slightly higher than that reported earlier (8). Values for OPRT activity in human leukocytes have not been reported previously. The absence of a striking increase of OPRT and ODC activities in leukocytes after allopurinol administration is consistent with the theory of enzyme stabilization because of the short life-span of these cells.

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CHAPTER 8

PYRIMIDINE METABOLISM IN ERYTHROCYTES OF THE NEWBORN

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8.1. SUMMARY

Activities of orotate phosphoribosyltransferase and orotidine 5'-phosphate decarboxylase were found to be significantly higher in erythrocytes from newborn infants than in erythrocytes from adults, and approximated those observed in patients with deficiency of hypoxanthine-guanine phosphoribosyltransferase. Enzyme activities were increased to a varying extent in patients with reticulocytosis. The results are discussed in relation to red cell age and stabilization of the enzymes by phosphoribosylpyrophosphate.

Pyrimidine 5'-nucleotidase was assayed by a new radiochemical method involving thin-layer chromatography for separation of product from substrate. Enzyme activity was higher with orotidine monophosphate than with uridine monophosphate. The activity of this enzyme was similar in erythrocytes of newborns and adults.

8.2. INTRODUCTION

Orotate phosphoribosyltransferase (OPRT, EC 2.4.2.10) and orotidine 5'-phosphate decarboxylase (ODC, EC 4.1.1.23) catalyze the final steps of pyrimidine biosynthesis de novo. Both enzymes are deficient in all but one cases of hereditary orotic aciduria, an inborn error of metabolism which is associated with megaloblastic anemia and macrocytosis (2,8). Deficiencies of folic acid or vitamin B_{12} are other well-known causes of megaloblastic anemia and macrocytosis due to defects in nucleic acid synthesis. Macrocytosis is also observed in the neonatal period. The mean corpuscular volume is 115 μ^3 during the first weeks of life of the full term child (4). It

seemed interesting to investigate whether the neonatal macrocytosis could be associated with a disturbance of pyrimidine metabolism during this period, other than those caused by folic acid or vitamin B_{12} deficiency.

In a previous investigation (16) we observed that both OPRT and ODC activities in human hemolysate are very labile in vitro but can be stabilized by phosphoribosylpyrophosphate (PRPP). PRPP concentration is higher in erythrocytes of neonates than in those of adults (3) which may increase the stability of OPRT and ODC. Furthermore, the mean cell age of circulating erythrocytes is considerably lower in neonates than in adults (4). Since several enzymes have an increased activity in young erythrocytes (4,6,9) a study of OPRT and ODC activity in erythrocytes of newborn children appeared to be an interesting approach.

Uridine monophosphate (UMP) which is the product of the consecutive action of OPRT and ODC but also a degradation product of ribonucleic acid, may be degraded by pyrimidine 5'-nucleotidase in erythrocytes (20). Hereditary deficiency (20) and lead-induced inhibition (13) of this cytoplasmic enzyme are associated with hemolytic anemia. It is known that the mean half life of neonatal erythrocytes is only 20 to 30 days compared with 55 days for erythrocytes from the adult (4). The activity of pyrimidine 5'-nucleotidase in neonatal erythrocytes has not yet been determined. Comparative measurements of pyrimidine 5'-nucleotidase were therefore included in the present study, using UMP and orotidine monophosphate (OMP) as substrates.

8.3. MATERIALS AND METHODS

Venous blood samples were obtained from healthy adults, newborns (four to nine days old, born at term) and seven children with various diseases. Children I, II and III had megaloblastic anemia while child IV had autoimmune hemolytic anemia. Patient V had congenital hemolytic anemia, basophilic stippling of red cells, enlarged spleen and reticulocytosis (reticulocyte count ranged from 4 to 11%). These findings were suggestive of deficiency of pyrimidine 5'-nucleotidase (20). Patients VI and VII were deficient in hypoxanthine-guanine phosphoribosyltransferase (HGPRT, EC 2.4.2.8) and did not receive allopurinol medication.

Erythrocytes were isolated by centrifugation, washed twice with 50 mmol/l Tris-HCl (pH 7.4) containing 100 mmol/l NaCl and lysed by freezing and

thawing twice.

OPRT and ODC were assayed by measuring $^{14}CO_2$ released from [carboxyl-^{14}C]orotic acid and [carboxyl-^{14}C] orotidine monophosphate, respectively. Details have been described elsewhere (15).

Pyrimidine 5'-nucleotidase was assayed by incubating hemolysate (0.4 - 1.0 mg of protein), Tris-HCl (28 mmol/l, pH.7.4) and either UMP-^{14}C (2.2 mmol/l, 1.1 mCi/mmol) or OMP-^{14}C (2.2 mmol/l, 0.5 mCi/mmol) in a final volume of 45 µl for 90 min at 37 °C. Reaction was terminated by heating for 5 min at 95 °C. Carrier uridine or orotidine was added and 10 µl samples were analysed by thin-layer chromatography using polyethyleneimine-cellulose (14). Distilled water was used in the separation of uridine and UMP while 0.2 mol/l LiCl was suitable to separate orotidine from OMP. Compounds were localised under ultraviolet light and eluted with 1 ml of 0.1 mol/l HCl / 0.2 mol/l KCl. Radioactivity was determined with 10 ml of Aquasol.

Spectral analysis of erythrocytes was performed according to Valentine et al. (20). Protein was determined according to Lowry et al. (11). Enzyme activities are expressed as nmoles per hr per mg protein. Statistical analysis was performed using the paired one-tailed Student's t-test.

8.4. RESULTS

Activities of both OPRT and ODC were found to be higher in erythrocytes from neonates than in erythrocytes from adults (Table 8.1). The difference is highly significant ($p < 0.001$). The ratio of ODC to OPRT activity is similar in erythrocytes from both groups. At about 3 months of age enzyme activities are within the range for erythrocytes from adults (Table 8.1). OPRT and ODC activities were higher in erythrocytes from children with deficiency of HGPRT than in erythrocytes from controls. Increased enzyme activities were also measured in erythrocytes from anemic patients (I to V) with a large variation in reticulocyte counts (normal reticulocyte count at this age is below 2%). The increase of enzyme activities seems to correlate with the percentage of reticulocytes (Table 8.1).

Spectral analysis at 240-300 nm of erythrocyte extracts from patient V revealed no abnormality in erythrocyte nucleotide pool. Maximal absorption was measured at 258 nm, as is observed with extracts of control erythrocytes.

Measurement of pyrimidine 5'-nucleotidase in erythrocytes from patient

TABLE 8.1.

ACTIVITIES OF OROTATE PHOSPHORIBOSYLTRANSFERASE AND OROTIDINE -5'-PHOSPHATE DECARBOXYLASE IN HUMAN HEMOLYSATE.

	OPRT	ODC
Adults (n=26)	0.22 ± 0.08	0.52 ± 0.20
Children 2-5 months (n=3)	0.14 ± 0.05	0.36 ± 0.10
Newborns (n=9)	0.77 ± 0.32	1.66 ± 0.28
Reticulocytosis		
Patient I (2.1)	0.49	1.01
Patient II (2.7)	1.40	1.32
Patient III (7.0)	1.70	2.63
Patient IV (12.4)	3.84	5.25
Patient V (3.8)	0.59	1.52
HGPRT deficiency		
Patient VI	0.73	2.17
Patient VII	1.00	1.70

Enzyme activities ± S.D in nmoles/hr per mg protein were determined as described in "Materials and Methods". The figures for erythrocytes from adults were taken from reference 15 Percentage of reticulocytes is given in parentheses for the patients I-V.

V yielded enzyme activities of 10.54 and 15.45 nmoles/hr per mg protein with UMP and OMP as substrates, respectively. These values are higher than the values found in erythrocytes from healthy adults (Table 8.2). No difference was found in pyrimidine 5'-nucleotidase activity of erythrocytes from newborns and adults (Table 8.2). Measurements of enzyme activity were performed without addition of Mg^{++} since this did not influence enzyme activity. Kinetic studies with respect to OMP as a substrate yielded a K_m value of 0.52 ± 0.15 mmol/l (mean ± S.D. of 5 experiments). OMP appeared to be a better substrate for pyrimidine 5'-nucleotidase assay than UMP, since enzyme activity was about 50% higher.

TABLE 8.2.

ACTIVITY OF PYRIMIDINE -5'-NUCLEOTIDASE IN ERYTHROCYTES FROM NEWBORNS AND ADULTS.

	Substrate	
	UMP	OMP
Adults (n=6)	5.9 ± 2.4	8.9 ± 3.2
Newborns (n=5)	6.2 ± 2.8	10.4 ± 3.5

Enzyme activity ± S.D. in nmoles/hr per mg protein was measured as described in "Materials and Methods".

8.5. DISCUSSION

The OPRT and ODC activities in the erythrocytes of newborn infants are significantly higher than those of the adult and comparable to those of patients with reticulocytosis (Table 8.1) When human erythrocytes were separated according to cell age, OPRT and ODC activities were higher in young cells than in older ones (7). After induction of reticulocytosis in the rabbit by acetophenylhydrazine, OPRT and ODC activities decreased rapidly after initial high enzyme activities (10). The mean cell age of neonatal erythrocytes is younger than that of adult erythrocytes (4). These findings suggest that the increased activities of OPRT and ODC in erythrocytes of the newborn reflect a young population of cells. A similar association of young cell age and high enzyme activity in erythrocytes has previously been reported for several enzymes of glucose metabolism (9) and purine metabolism (3,6). The specific activity of adenine phosphoribosyltransferase was positively correlated with the reticulocyte percentage (6).

Another factor which may contribute to the increased OPRT and ODC activities in neonatal erythrocytes is stabilization of the enzymes by PRPP. The concentration of PRPP is significantly higher in erythrocytes of neonates than in those of adults (3). OPRT and ODC in human hemolysate are

stabilized in vitro by PRPP (16). The increased activities of OPRT and ODC in erythrocytes from patients with deficiency of HGPRT (1, 16, Table 8.1) may be ascribed to stabilization by PRPP. PRPP concentration is increased in erythrocytes of these patients (5, 17). It has been argued that the increased activity of OPRT and ODC in these erythrocytes is not due to stabilization by PRPP. The evidence against stabilization by PRPP was mainly based on thermal inactivation experiments (1). Since PRPP is very unstable at elevated temperatures especially in the presence of hemolysate and Mg^{++}-ions (17), such experiments do not seem appropiate to investigate stabilization by this compound. Stabilization of enzyme activity by PRPP has also been postulated to explain the increased activity of adenine phosphoribosyltransferase in erythrocytes from neonates and from patients with deficiency of HGPRT (3).

Pyrimidine 5'-nucleotidase is usually assayed by measuring the release of inorganic phosphate from UMP, CMP or TMP (12, 20). A radiochemical assay has been described which was based on precipitation of CMP (18). We employed a simple chromatographic procedure to separate product from substrate. OMP proved to be the substrate that gave the highest enzyme activity, but the K_m value measured for OMP is higher than that observed for CMP and UMP (12, 19). OMP can also be decarboxylated by ODC present in the hemolysate, but it can be calculated from the figures given in Tables 8.1 and 8.2 that the decrease of substrate concentration caused by ODC activity is negligible. No significant difference in pyrimidine 5'-nucleotidase activity (with either OMP or UMP as substrate) was observed between neonatal erythrocytes and erythrocytes from adults (Table 8.2). Since it was reported that this enzyme activity decreased with the age of the red cell (19) one might expect an increased enzyme activity in neonatal erythrocytes which have a young mean cell age. Apparently there is no rapid decrease of enzyme activity with increasing cell age. Consistent with this is the observation that pyrimidine 5'-nucleotidase in erythrocytes from patients with marked reticulocytosis is only two-fold increased when compared with normal erythrocytes (12, 20). We also measured a slight increase of the activity of this enzyme in patient V with reticulocytosis.

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CHAPTER 9

INHIBITION OF OROTATE PHOSPHORIBOSYLTRANSFERASE AND OROTIDINE 5'-PHOSPHATE DECARBOXYLASE OF HUMAN ERYTHROCYTES BY PURINE AND PYRIMIDINE NUCLEOTIDES

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9.1. SUMMARY

Orotidine 5'-phosphate decarboxylase of human hemolysates exhibits triphasic kinetics with K_m values of 33, 1.7 and 0.082 μM. Inhibition of this enzyme at low OMP concentrations (< 3 μM) by several naturally occurring purine and pyrimidine nucleotides was investigated. No significant inhibition was observed with IMP, GMP, TMP, ADP, ATP and TTP at 5 mM. Inhibition constants for CMP, AMP and dAMP were 31 μM, 0.11 mM and 0.21 mM, respectively. The results are discussed in relation to inhibition by nucleotides of orotate phosphoribosyltransferase, previously measured with a method which depends on orotidine 5'-phosphate decarboxylase activity.

9.2. INTRODUCTION

Orotate phosphoribosyltransferase (EC 2.4.2.10) and orotidine 5'-phosphate decarboxylase (EC 4.1.1.23) catalyze the conversion of orotic acid to UMP in de novo pyrimidine biosynthesis:

$$\text{orotate} + \text{PRPP} \xleftarrow{Mg^{2+}} \text{OMP} + PP_i$$

$$\text{OMP} \longrightarrow \text{UMP} + CO_2$$

ODC activity is inhibited by its product UMP (1-3) and by ribonucleotides of several purine and pyrimidine analogues, e.g. azauridine (4,5), allopurinol (3, 5-7) and oxipurinol (5-7). Conflicting evidence has been presented with respect to inhibition of ODC by naturally occurring nucleotides (see Discussion). We demonstrated that OPRT activity in human hemolysates is also inhibited by its product, OMP. The inhibition was competitive with respect

to PRPP (7). Furthermore, several purine and pyrimidine nucleotides appeared to be inhibitors of OPRT activity without affecting ODC activity (7). Traut and Jones (8) have criticized this latter conclusion. Since OPRT activity was assayed by measuring the release of $^{14}CO_2$ from [carboxyl-^{14}C]orotic acid, the assay was dependent on the ODC activity and inhibition of ODC activity would be mistakenly interpreted as inhibition of OPRT activity. Prior to testing the effects of nucleotides on OPRT activity their non-interference with the ODC reaction was ascertained at 0.1 mM OMP (7). During the OPRT assay OMP concentration is, however, much lower and inhibition of ODC by nucleotides might be significant (8). We therefore decided to investigate in detail the inhibition of ODC activity in human hemolysates by purine and pyrimidine nucleotides at low concentrations of OMP (< 3 μM). Special attention was given to the possibility that derivatives of adenosine (AMP, ADP, ATP, dAMP) inhibit the conversion of orotic acid to UMP. Inhibition of OPRT (9) or ODC (8) by AMP has been suggested as the mechanism responsible for inhibition of cell growth by adenosine. Deficiencies of enzymes of adenosine catabolism (adenosine deaminase deficiency (10) and purine nucleoside phosphorylase deficiency (11)) are associated with immunodeficiencies.

9.3. MATERIALS AND METHODS

The procedures for measuring the activities of OPRT and ODC in human hemolysates using [carboxyl-^{14}C]labeled substrates were described before (7). Freshly prepared hemolysates were used for all experiments. ODC was assayed at 0.2 - 3 μM substrate concentration with [carboxyl-^{14}C]orotidine 5'-monophosphate of high specific activity (37 mCi/mmol). All inhibitors tested were present at a final concentration of 5 mM except for CMP which was present at 2.5 mM. Care was taken to maintain the pH at 7.4 when nucleotides were present. Radioactivity in samples of [carboxyl-^{14}C]orotic acid and [carboxyl-^{14}C]orotidine 5'-monophosphate was measured in the presence of 0.2 ml of hydroxide of Hyamine (Packard) to trap any $^{14}CO_2$ released into the scintillation fluid.

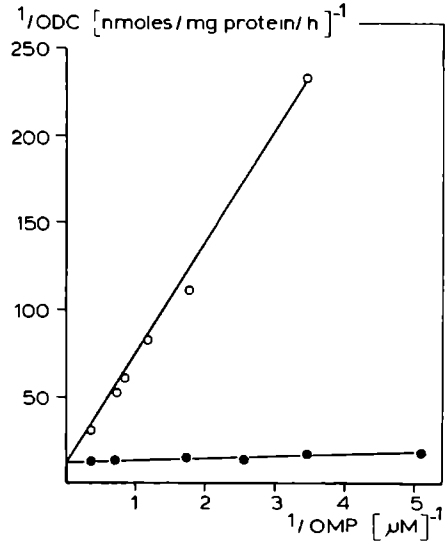

Fig. 9.1. Inhibition of ODC in human hemolysate by CMP. Control (●), 2.5 x 10^{-3}M CMP (o).

At OMP concentrations below 3 μM a K_m value of 0.082 ± 0.024 μM (mean ± S.D., 8 determinations) was found. All compounds previously reported as inhibitors of OPRT in hemolysates (7), and in addition dAMP, were tested for inhibition of ODC at 2 μM OMP(Table 9.1).Only CMP, AMP and dAMP caused a significant inhibition, which was competitive with respect to OMP. Fig.1 illustrates the inhibition by CMP. The K_1 value for CMP was lower than for AMP or dAMP(Table 9.1).PRPP at 1 mM did not affect the activity of ODC or the inhibition of ODC by CMP, AMP and dAMP.

At a high concentration of PRPP (0.8 mM) OPRT activity was inhibited by TTP but not by AMP, ADP, IMP or TMP and only slightly (< 15%) by ATP or GMP. At a non-saturating concentration of PRPP (0.2 mM) AMP, ADP, ATP, IMP, GMP, TMP and TTP inhibited OPRT activity as earlier reported (7). The amount of $^{14}CO_2$ measured in the assay of OPRT activity with [^{14}C-carboxyl]-orotic acid could not be increased under these inhibitory conditions by a prolongation of the second incubation period after injection of EDTA. Since EDTA injection only stops the OPRT reaction by complexing the Mg^{2+} ions,

TABLE 9.1

INHIBITION OF ERTHROCYTE OROTIDINE-5'-PHOSPHATE DECARBOXYLASE AT LOW CONCENTRATION OF OMP

Competitive inhibitor	K_i value
CMP	31 ± 4 μM
AMP	0 11 ± 0.03 mM
dAMP	0 21 ± 0 04 mM

The OMP concentration ranged from 0 2 - 3 μM The K_m value for OMP was 0 082 ± 0 024 μM (mean ± S D of 8 determinations) K_i values are the mean ± S D of 3-6 determinations The following compounds were not inhibitory when tested at a final concentration of 5 mM and an OMP concentration of 2 μM GMP, IMP, TMP, ADP, ATP, TTP

but does not affect ODC activity, a prolongation of this second incubation period would relieve inhibition of ODC activity, which could decrease $^{14}CO_2$ production. This was not the case, which indicates that the inhibition is on the level of OPRT, not on that of ODC.

9.5. DISCUSSION

Previously we found bimodal kinetics for the ODC enzyme in human hemolysates with K_m values of 33 and 1.7 μM (7). At low concentrations of OMP (< 3 μM) we now found a third K_m value of 0.082 μM. Brown et al. (12) demonstrated that purified ODC from human erythrocytes exists in three forms, corresponding to monomer, dimer and tetramer, with K_m values of 25, 3 and 0.6 μM, respectively. It appears therefore that the K_m values of 33, 1.7 and 0.082 μM measured in crude hemolysates correspond to monomer, dimer and tetramer forms of the enzyme. The difference in K_m values between purified enzyme and crude hemolysate may be related to the purification procedure. The enzyme from human liver was also found to exist in monomer, dimer and tetramer forms (13). The kinetic properties of the individual forms appeared identical, possibly as a result of interconversion during the assay. The K_m

value was 0.12 μM (13). Fyfe et al. (6) found biphasic kinetics for the ODC enzyme from rat liver with K_m values of 4 and 1 μM. Two K_m values (4.5 and 0.33 μM) were also reported for the ODC enzyme from human fibroblasts (14).

Inhibition of ODC activity by CMP was also observed for the enzyme from cow brain (2) and human liver (13) with K_i values of 0.14 mM and 40 μM, respectively. AMP and GMP were also effective inhibitors of cow brain ODC when present at 5 mM (2). ODC from human hemolysate was not inhibited by IMP or GMP and only slightly by AMP at 5 μM OMP (3). The tetramer form of ODC, purified from human hemolysate by Brown and O'Sullivan (5) was inhibited for less than 10 per cent by 5 μM AMP, ADP, ATP, GMP, TMP or CMP at 0.5 μM OMP. The results of inhibition studies on the OPRT/ODC enzyme complex from Ehrlich ascites cells (8) seem to be completely opposite to our data. AMP, ADP, IMP, TMP and TTP were found to have little effect on the OPRT activity while being inhibitors of the ODC activity. The conditions used in these experiments, however, were markedly different from the ones we applied. ODC activity was measured at 0.1 μM OMP which is much lower than the K_m value (0.8 μM) of the enzyme preparation used (15) while OPRT activity was assayed at a saturating concentration of PRPP. Inhibition of OPRT from human hemolysate is, however, competitive with PRPP for all inhibitors except TTP as was stated before (7) and confirmed in the present investigation. Inhibition of ODC activity cannot be the cause of the measured decrease in $^{14}CO_2$ production from [carboxyl-^{14}C]orotic acid in the presence of purine and pyrimidine nucleotides, as suggested by Traut and Jones (8).

The K_i values with respect to ODC activity, measured for the naturally occurring nucleotides CMP, AMP and dAMP are high both in comparison to their physiological concentration and in comparison to the K_m value for OMP. Thus, the inhibition of ODC by these nucleotides does not seem to be of physiological significance. The intracellular concentration of PRPP is well below the K_m value of OPRT for this compound (16). Inhibition of OPRT which is competitive with respect to PRPP may be important for the in vivo regulation of pyrimidine biosynthesis and deserves further investigation.

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CHAPTER 10

ACTIVITY OF ADENOSINE DEAMINASE AND PURINE NUCLEOSIDE PHOSPHORYLASE IN ERYTHROCYTES AND LYMPHOCYTES OF MAN, HORSE AND CATTLE

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10.1. SUMMARY

1. Activities of ADA and PNP were measured in erythrocytes and lymphocytes of man, horse and cattle.
2. In bovine hemolysates both enzyme activities are low when compared with activities in human hemolysates. In horse hemolysates both enzyme activities are virtually absent.
3. Enzyme activities are consistently lower (about 50%) in intact lymphocytes than in sonicated lymphocytes. This finding suggests that the uptake of nucleosides is rate-limiting for both enzymes in intact lymphocytes.
4. The activity of ADA in horse lymphocytes is comparable to that in lymphocytes of patients with severe combined immunodeficiency associated with ADA deficiency.

10.2. INTRODUCTION

Deficiency of adenosine deaminase (ADA) or purine nucleoside phosphorylase (PNP) in man is associated with defects of cell-mediated immunity (Giblett et al., 1972, Giblett et al., 1975; Parkman et al., 1975). In horse and cattle erythrocytes ADA activity is virtually absent (McGuire et al., 1976; Van Belle, 1969). A low activity of PNP was reported for bovine erythrocytes (Duhm, 1974). When ADA and PNP activities in lymphocytes from horse and cattle would also be low, these cells might provide a suitable system for studying the metabolic disturbances caused by deficiency of ADA or PNP. Previously we reported large differences in activities of several other

enzymes of purine and pyrimidine metabolism between erythrocytes of ten mammalian species (Tax et al., 1976; Tax and Veerkamp, 1978). Now we found large variations in ADA and PNP activity of erythrocytes and lymphocytes between man, horse and cattle.

10.3 MATERIALS AND METHODS

Enzyme preparations

Heparinized blood samples were obtained from adult human volunteers, from horses (healthy adults) and from cattle (local slaughterhouse). Erythrocytes were isolated by centrifugation, washed twice with Tris-buffered saline (pH 7.4) and lysed by adding 3 vol 10 mM Tris (pH 7.4). Lymphocytes were isolated from peripheral blood using Ficoll-Isopaque according to Böyum (1968). Cells were counted with a hemocytometer and lysed by sonication (Branson sonifier, 8 periods of 5 sec at maximal output). Intact or sonified lymphocytes as indicated were used for all enzyme assays immediately upon preparation. Freshly prepared hemolysates were used for assays of ADA.

Enzyme assays

All enzymes were assayed at 37 ^{o}C by radiochemical methods. Enzyme activities are expressed as nmol/h per 10^6 lymphocytes or for erythrocytes as nmol/h per mg protein. Protein was measured according to Lowry et al. (1951). Isotonic conditions were maintained during incubations with intact lymphocytes.

Adenosine deaminase assay

The reaction mixture (60 µl) of lymphocytes contained 40 mM phosphate buffer (pH 7.4), 110 mM NaCl, 0.16 mM [8-^{14}C]adenosine (49 mCi/mmol) and the equivalent of 0.025-0.25 x 10^6 cells. Hemolysates were assayed by incubating hemolysate protein (60-3000 µg) with 50 mM Tris-HCl buffer (pH 7.4), 1 mM EDTA and 0.16 mM [8-^{14}C]adenosine in a final volume of 60 µl. After 30 min reaction was terminated by boiling for 2 min and addition of excess carrier adenosine, inosine and hypoxanthine. Separation was achieved by high voltage paper electrophoresis with 40 mM sodium borate (pH 8.65) containing

1 mM EDTA (1 h at 70 V/cm). Spots were localized under ultraviolet light and eluted with 5 ml of 0.1 M NaOH. Radioactivity was determined in 10 ml of Aquasol (New England Nuclear, Dreieichenhain, W.Germany).

Purine nucleoside phosphorylase assay

Assay conditions in lymphocytes were the same as for ADA except that 0.35 mM [8-^{14}C]inosine (9 mCi/nmol) replaced adenosine and total volume was 50 µl. Incubation mixture for measuring purine nucleoside phosphorylase in hemolysates contained 30 mM Tris-HCl (pH 7.4), 1 mM EDTA, 100 mM phosphate (pH 7.4) and 0.35 mM [8-^{14}C]inosine in a volume of 50 µl. Incubations were terminated after 10 min by boiling for 2 min and addition of carrier inosine and hypoxanthine. Substrate and product were separated by thin-layer chromatography on polyethyleneimine-cellulose using distilled water as developer and eluted with 1 ml 0.1 M HCl/0.2 M KCl. Radioactivity was determined after addition of 10 ml of Aquasol.
Intact lymphocytes were preincubated in the phosphate-containing medium for 15 min before addition of inosine.

Animals

The following species were used: Man - *Homo sapiens*, Horse - *Equus caballus*, Cattle - *Bos taurus*.

10.4. RESULTS

With all enzyme assays, product formation was linear with time and protein concentration. In ADA assays, both inosine and hypoxanthine were formed due to the presence of PNP activity. No AMP was formed from adenosine by lymphocytes, and the addition of EDTA prevented the production of AMP in hemolysates. Hypoxanthine was the only product formed from inosine in PNP assays.

ADA activity in cattle hemolysate was low when compared with the activity in human hemolysate and was below detection limit in horse hemolysate (Table 10.1). Cattle hemolysate also showed a low PNP activity. PNP activity was still lower in hemolysate of horse. Species differences in PNP activity were much smaller in lymphocytes (Table 10.2). PNP and ADA activities were consistently lower (about 50%) in intact lymphocytes than in

TABLE 10.1

ADA AND PNP ACTIVITIES IN HEMOLYSATES OF MAN, CATTLE AND HORSE.

Species	ADA	PNP
Man	59 + 18	1249 + 222
Cattle	0.7 ± 0.7	39 ± 17
Horse	< 0.2	1.0 ± 0.5

Enzyme activities in nmol/h per mg protein. Values are the means ± S.D. of 3-5 determinations.

TABLE 10.2

ADA AND PNP ACTIVITIES IN LYMPHOCYTES OF MAN, CATTLE AND HORSE.

Species	ADA		PNP	
	Intact cells	Lysate	Intact cells	Lysate
Man	44 ± 15	102 ± 30	177 ± 48	385 ± 154
Cattle	21 ± 7	50 ± 18	48 ± 18	96 ± 12
Horse	4 ± 2	7 ± 2	42 ± 3	74 ± 22

Enzyme activities in nmol/h per 10^6 cells.

Values are the means ± S.D. of 4 determinations.

sonicated lymphocytes. ADA activity in horse lymphocytes was only a few percent of the value found in human lymphocytes, while cattle lymphocytes had an intermediate value.

10.5 DISCUSSION

Activities of ADA and PNP in human hemolysate (Table 10.1) are comparable to those reported by other investigators (Cartier and Hamet, 1976, Parkman et al., 1975, Scholar and Calabresi, 1973, Snyder et al., 1976). The low value of PNP activity in horse hemolysate has not been reported before. Castles et al. (1977) reported rather high values of both PNP and ADA activities in horse hemolysate. No significant ADA activity could be detected in horse hemolysate by McGuire et al. (1976). These authors also measured ADA activity in lymphocytes from normal horses and horses with combined immunodeficiency. In both groups ADA activity was similar to our values. They did not, however, compare ADA activity in horse lymphocytes with that in other mammalian lymphocytes. ADA activity in lysed human lymphocytes (Table 10.2) is similar to values measured by others in total lysate from lymphocytes (Cartier and Hamet, 1976) or in 20,000 g supernatant of sonic extracts (Snyder et al., 1976). However, PNP activity in total lysate (Table 10.2) seems to be much higher than the values reported for 20,000 g supernatants (Scholar and Calabresi, 1973, Snyder et al., 1976). This finding suggests that part of the lymphocyte PNP activity is membrane-associated, as is proposed for fibroblasts (Cohen and Martin, 1977). Both ADA and PNP activities are lower in intact lymphocytes than in sonicated lymphocytes (Table 10.2) probably because the uptake of nucleosides is rate-limiting (Snyder et al., 1976).

Deficiency of ADA in man is associated with immunodeficiency. A low ADA activity was found in lymphocytes of both normal horses and foals with combined immunodeficiency (McGuire, 1976). ADA activity is apparently not necessary for normal lymphocyte function in horses. Comparison of lymphocyte metabolism in man and horse may provide valuable information about the role of ADA in lymphocytes.

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CHAPTER 11

URIDINE METABOLISM IN LYMPHOCYTES AND ERYTHROCYTES OF MAN, HORSE AND CATTLE

11.1. SUMMARY

1. No conversion of uridine to uracil or uridine nucleotides was found in intact erythrocytes and in hemolysates of man, horse and monkey.
2. Uridine phosphorylase activity was high in lymphocyte extracts of man and cattle, but low in those of horse. Conversion of uridine to uracil was also found with intact lymphocytes from man but not with those from horse.
3. Both uridine kinase activity and incorporation of uridine into nucleotides and RNA were of the same order of magnitude when human lymphocytes were compared with those of horse.
4. Rates of phosphorylation and phosphorolysis of uridine in intact lymphocytes were only a few percent of the corresponding enzyme activities measured in lymphocyte extracts.
5. The results are discussed in relation to adenosine-induced pyrimidine starvation and the phenomenon that horses have a normal immunoresponse despite a low activity of adenosine deaminase in their lymphocytes.

11.2. INTRODUCTION

Pyrimidine nucleotides can be formed by de novo biosynthesis or by re-utilization of nucleosides, the salvage pathway. The relative contribution of both pathways to the total production of pyrimidine mucleotides is different in various types of cells. Uridine kinase (EC 2.7.1.48) plays a key role in the salvage pathway. The enzyme phosphorylates uridine and cytidine as well as a number of pyrimidine nucleoside analogues (1). Drug resistance may be associated with deficiency of uridine kinase (2). The enzyme is

induced in lymphocytes stimulated by phytohemagglutinin (3). Uridine can also be catabolized to uracil by uridine phosphorylase (EC 2.4.2.3). Catabolism of uridine may be significant as evidenced by the rapid conversion to uracil in the blood of rat (4), mouse (5) and dog (6).

In a previous investigation (7) we found that the activity of adenosine deaminase (ADA, EC 3.5.4.4) in horse lymphocytes is comparable to that in lymphocytes of patients with severe combined immunodeficiency associated with ADA deficiency (8). The mechanism by which the enzyme deficiency leads to immunodeficiency is still unknown, but there is ample evidence for interference of pyrimidine synthesis de novo (9-11). When ADA deficiency leads to interference of pyrimidine synthesis de novo, such an interference could be bypassed in horse lymphocytes when pyrimidine nucleotides are formed preferentially by the salvage pathway. Horse lymphocytes have a low activity of orotidine 5'-phosphate decarboxylase (ODC, EC 4.1.1.23), the final enzyme of the de novo pathway (12). We now studied the metabolism of uridine in lymphocyte extracts of man, horse and cattle as well as in intact lymphocytes of man and horse.

Horse and human erythrocytes were also investigated with respect to their uridine metabolism since we found previously (13) marked differences between both species in the activities of orotate phosphoribosyltransferase (OPRT, EC 2.4.2.10) and ODC.

11.3. MATERIALS AND METHODS

Materials

Ficoll (MW 400,000) was obtained from Pharmacia and Isopaque from Nyegaard & Co., Oslo, Norway. A Ficoll-Isopaque solution was prepared by dissolving 9.56 g Ficoll in 130 ml distilled water and adding 20 ml Isopaque; distilled water was added until a final specific gravity of 1.077 was obtained. Minimum essential medium for suspension cultures (MEMS), human serum and horse serum were obtained from Flow Lab. [2-^{14}C]uridine was purchased from the Radiochemical Centre, Amersham and hydroxide of Hyamine 10-X from Packard. Thin-layer plates containing polyethyleneimine-cellulose (PEI-cellulose) were obtained from Merck. Omnifluor was purchased from New England Nuclear Corp.

Preparation of cell suspensions and cell extracts

Blood samples from human adults, healthy adult horses and cattle were collected in heparinized tubes. Erythrocytes were isolated by centrifugation, washed twice with 50 mM Tris/HCl (pH 7.4) containing 100 mM NaCl, and resuspended in a solution containing 40 mM potassium phosphate (pH 7.4), 110 mM NaCl, 5.5 mM glucose and 1 mM $MgCl_2$. Lymphocytes were isolated at room temperature by Ficoll-Isopaque gradient centrifugation of diluted blood (14) and counted in a hemocytometer. For experiments with intact lymphocytes cells were suspended in a mixture of 10% homologous serum and 90% MEMS containing 2 mM glutamin and penicillin (100 U/ml) and streptomycin (100 µg/ml).

Hemolysates were prepared by freezing and thawing the erythrocytes twice. Extracts of lymphocytes were obtained by sonication (Branson sonifier, 8 bursts of 5 sec at maximal output) in 50 mM Tris-HCl (pH 7.4) containing 1 mM EDTA and centrifugation at 7,000 g (15 min, 4 ^{o}C).

Enzyme assays

Uridine kinase and uridine phosphorylase activities in hemolysates and lymphocyte extracts were assayed at 37 ^{o}C by radiochemical methods. Enzyme activities are expressed in nmol/hr per 10^6 cells.Linearity of the reactions with respect to time and amount of cell extract was ascertained. The incubation mixture (70µl) for uridine kinase assay contained 40 mM Tris-HCl (pH 7.4), 1 mM EDTA, 10 mM $MgCl_2$, 5 mM ATP and 70 µM [2-^{14}C]uridine (55 mCi/mmol). Reaction was terminated after 60 min by heating the incubation mixture at 95 ^{o}C for 5 min (Eppendorf heater). After centrifugation and addition of carriers, uracil and uridine nucleotides were separated from uridine by thin-layer chromatography (TLC) on PEI-cellulose (15). Assay conditions for uridine phosphorylase were the same as for uridine kinase except that 40 mM orthophosphate (pH 7.4) was added.

Metabolism of uridine by intact cells

Suspensions of erythrocytes (25%, by vol) or lymphocytes (30 x 10^6 cells/ml) were preincubated for 30 min at 37 ^{o}C with gentle shaking. [2-^{14}C]uridine was then added to a final concentration of 70 µM. After appropriate times (up to 3 hr) 50 µl samples were taken and mixed with 10 µl 10 mM uridine/10 mM uracil. Cells were separated from the medium by centrifugation at

3,500 g (5 min 4 °C, Burkard Koolspin centrifuge) and the cells were washed with 50 µl saline. Medium and wash fluid were combined, deproteinized, and analyzed by means of TLC on PEI-cellulose. Cells were extracted with 50 µl 0.5 N $HClO_4$ and acid-insoluble material was washed once with 50 µl 0.5 N $HClO_4$. Centrifugation was at 9,500 g (5 min, 4 °C). Extract together with the wash fluid was neutralized with 5 N KOH and after centrifugation of $KClO_4$ samples were analyzed on TLC sheets coated with PEI-cellulose. 0.2 ml of 1 N hydroxide of Hyamine was added to the acid-insoluble cell material and left overnight at room temperature. Radioactivity was then counted with 10 ml of toluene containing 4 g of omnifluor per liter.

11.4. RESULTS

After incubation of erythrocytes of man, horse or Rhesus monkey with [2-^{14}C]-uridine, no significant radioactivity was found in either uracil or uridine nucleotides. Virtually all radioactivity was recovered in uridine. Hemolysates from these three species also showed no conversion of uridine.

Uridine kinase activity is similar in lymphocyte extracts of man, cattle and horse (Table 11.1). Uridine phosphorylase activity is higher than

TABLE 11.1.

ACTIVITIES OF URIDINE KINASE AND URIDINE PHOSPHORYLASE IN LYMPHOCYTE EXTRACTS OF MAN, CATTLE AND HORSE

Species	Uridine kinase	Uridine phosphorylase
Man	0.51 ± 0.15 (6)	2.13 ± 0.32 (4)
Cattle	0.48 ± 0.35 (8)	1.90 ± 0.91 (4)
Horse	0.57 ± 0.15 (6)	0.33 ± 0.19 (4)

Enzyme activities are given in nmol/hr per 10^6 cells as the means ± S.D. Numbers in parentheses refer to the number of individuals. Extracts from lymphocytes were prepared as described in Materials and Methods.

TABLE 11.2.

URIDINE METABOLISM IN INTACT LYMPHOCYTES OF MAN AND HORSE

Species	Phosphorylation		Phosphorolysis
	Nucleotides	RNA	Uracil
Man	1860 ± 199 (4)	974 ± 172 (4)	7661 ± 1539 (3)
Horse	1970 ± 462 (4)	1123 ± 63 (4)	< 1000 (4)

Intact lymphocytes were incubated with 70 μM [2-^{14}C] uridine (55 mCi/mmol) for periods up to 3 hr. Radioactivity in cells and medium was analyzed as described in Materials and Methods. The conversion of uridine to nucleotides or uracil and the incorporation into RNA are given in dpm/hr per 10^6 cells. Values are the means ± S.D. of 3-4 experiments in quadruplicate.

uridine kinase activity in lymphocyte extracts from man and cattle, but lower in lymphocyte extracts from horse.

When intact lymphocytes from man and horse were incubated with uridine, product formation was linear with time for at least 2 hr. No conversion of uridine could be detected after incubation with a mixture of 90% MEMS and 10% human serum or horse serum. A significant amount of uracil was found in the medium after incubation of human lymphocytes with uridine but not after incubation of horse lymphocytes. Both with human and horse lymphocytes most of the intracellular acid-soluble radioactivity was associated with uridine nucleotides. Radioactivity was also present in the acid-insoluble material which is assumed to contain the cellular RNA. Table 11.2 shows that both the conversion of uridine to uridine nucleotides and its incorporation into RNA are of the same order of magnitude in human and horse lymphocytes. The amount of uridine which is metabolized by intact lymphocytes (Table 11.2) can be calculated in nmol/hr per 10^6 cells from the specific activity of uridine (122,000 dpm/nmol) when the original intracellular uridine pool is neglected. Both the rate of phosphorylation (about 24 pmol/hr per 10^6 cells) and of phosphorolysis (about 62 pmol/hr per 10^6 cells in human lymphocytes and < 8 pmol/hr per 10^6 horse lymphocytes) are only a few percent of the enzymatic activities measured in lymphocyte extracts.

Human erythrocytes apparently do not phosphorylate (or cleave) uridine, as was also reported by others (16). The presence of uridine kinase in erythrocytes from Rhesus monkey has been reported (17) but we could not confirm this report. Horse erythrocytes also lack uridine kinase which is remarkable since two enzymes of the de novo pathway, OPRT and ODC, are also absent in horse erythrocytes (13). Therefore UMP can also not be synthesized from exogenous orotic acid in horse erythrocytes and must be either conserved during the life span of the erythrocytes or supplied by some unknown alternative pathway.

The much slower rate of metabolism of uridine in intact lymphocytes in comparison to that in lymphocyte extracts may be related to cell permeability, to availability of other substrates or to cellular regulation systems. The low intracellular concentration of orthophosphate may limit the activity of uridine phosphorylase. The activity of intracellular uridine kinase may be restricted by the concentration of ATP and Mg^{2+}. A more important factor is probably the uridine uptake through the cell membrane of the lymphocyte (18). The activity of uridine kinase is subject to inhibition by pyrimidine nucleotides in cell extracts (1), but there is no evidence for this feedback control in intact cells.

The activity of uridine kinase found in human and bovine lymphocytes is comparable to values reported (18-20). (The extract of 10^7 human lymphocytes was equivalent to about 1 mg of protein).

The presence of uracil in the medium after incubation of intact human lymphocytes with uridine does not necessarily implicate that phosphorolysis takes place at the cell membrane, though a membrane-associated uridine phosphorylase has been demonstrated in mouse fibroblasts (21) and rat liver (22). Usually only the radioactivity present in the cells is analyzed and the medium is discarded (18,23).

In horse lymphocytes the rate of catabolism of uridine is less than in lymphocytes of man and cattle and lower than the rate of phosphorylation (Tables 11.1,11.2).Furthermore, the activity of ODC is markedly lower than in lymphocytes from man and cattle (12). Horse lymphocytes apparently have a preference for the utilization of uridine to synthesize pyrimidine nucleotides. This may contribute to the fact that horses have a normal immunoresponse despite a low ADA activity in their lymphocytes (7). In this respect

it should be mentioned that uridine can reverse adenosine toxicity in fibroblasts (9) and lymphoma cells (10,11), but not in cells which are deficient in uridine kinase (24). Myoblast cells having a decreased activity of uridine kinase were more sensitive to the growth inhibitory effects of adenosine than the parental line (25).

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CHAPTER 12

SURVEY AND SUMMARY

12.1. THE SIGNIFICANCE OF PHOSPHORIBOSYLPYROPHOSPHATE

Phosphoribosylpyrophosphate plays a key role in pyrimidine and purine metabolism and their interrelationship. It is a substrate as well as an allosteric activator of both pyrimidine and purine synthesis de novo, and is also a substrate of the salvage pathway for the synthesis of purine nucleotides (see 1.3). PRPP is also involved in the conversion of allopurinol and oxipurinol to their ribonucleotide derivatives (see 1.4). The ribonucleotides of allopurinol and oxipurinol are the effectors of the allopurinol effects on pyrimidine metabolism (see below, 12.2). The synthesis of PRPP may be related to the catabolism of adenosine and inosine through the intermediates ribose 1-phosphate and ribose 5-phosphate (Fig. 1.8). The inhibition of pyrimidine biosynthesis de novo secondary to deficiency of ADA or PNP is probably associated with a decrease of PRPP concentration (see 1.5).

The first part of this thesis (chapter 2-5) is therefore devoted to a study of the concentration and metabolism of PRPP in mammalian erythrocytes. Special attention is given to the effects of inherited enzyme deficiencies.

In chapter 2 we report on the activities of phosphoribosyltransferases (OPRT, APRT and HGPRT) in hemolysates of ten mammalian species. These enzymes are responsible for the utilization of PRPP in erythrocytes. Both HGPRT and OPRT activities were virtually absent in horse hemolysates. The deficiency of HGPRT was confirmed in studies with intact horse erythrocytes.

In chapter 3 a simple and sensitive assay is described for estimating the concentration and rate of synthesis of PRPP. The procedure is based on the release of $^{14}CO_2$ from [carboxyl-^{14}C]orotic acid in the presence of a preparation of OPRT and ODC. In this way the total product of the PRPP-dependent reaction can be measured, in contrast to procedures involving thin-layer chromatography or electrophoresis of small samples from the assay mixture. Using this assay an increased PRPP concentration was measured in erythrocytes from patients with partial or complete deficiency of HGPRT. The

increased PRPP concentration appears to result from a decreased utilization of PRPP, since the activity of PRPP synthetase was normal. A patient with a deficiency of PNP also had an increased erythrocyte PRPP concentration which may also be ascribed to a decreased utilization of PRPP since this enzyme deficiency blocks the formation of hypoxanthine, the substrate of HGPRT. PRPP synthetase activity was found to be normal in erythrocytes from all subjects studied.

In chapter 4 the occurrence of inherited abnormalities of PRPP metabolism in hyperuricemia and gout was investigated. An increased availability of PRPP may cause an accelerated rate of purine biosynthesis de novo and overproduction of uric acid, as discussed in 1.3. This investigation included measurements of PRPP synthetase activity at suboptimal concentrations of the substrates ATP and ribose 5-phosphate in order to detect abnormalities in substrate affinity. No such kinetic variants were detected in erythrocytes of 12 adults with primary gout and of 50 adolescents with a high concentration of urate in serum. The concentration of PRPP in erythrocytes was normal in all subjects studied. No striking increases of PRPP synthetase activity were found in the erythrocytes of any subject, but the group of adolescents with a high serum urate concentration had a significantly higher activity of PRPP synthetase than the control group. This finding may be relevant to the pathogenesis of gout since the late onset of this disease may be due to a slow accumulation of uric acid. It seems worthwhile to investigate PRPP metabolism in other cell types from hyperuricemic and gouty patients. Lymphocytes or cultured fibroblasts seem to be more appropriate than the anucleate erythrocyte which lacks the de novo pathway for purine synthesis.

In chapter 5 the concentration of PRPP in hemolysates of ten mammalian species is compared with the capacities of the enzymes responsible for the synthesis and utilization of this compound. Actual enzyme activities in vivo are modulated by the availability of substrates and the concentration of regulatory compounds, and will usually be much lower than maximal capacities. Some correlations, however, between enzyme activities and PRPP concentration seem to hold. Low activities of HGPRT were consistently associated with high concentrations of PRPP, e.g. in erythrocytes of patients with a deficiency of HGPRT (chapter 3) and in horse erythrocytes (chapter 5). In cattle erythrocytes there was a high activity of HGPRT together with a high concentration of PRPP. The activity of PNP, however, was extremely low in

these erythrocytes and therefore the formation of the HGPRT substrate hypoxanthine was absent. The high concentration of PRPP in cattle erythrocytes therefore appears to result from a decreased utilization (cf. deficiency of PNP in man, chapter 3). In chapter 5 we also report on an alternative route for the catabolism of PRPP. The enzymatic degradation of PRPP observed in mammalian hemolysate seems to be associated with acid phosphatase activity. The activity of acid phosphatase was measured in hemolysates of ten mammalian species using p-nitrophenylphosphate as substrate. The activity of the enzyme appeared to correlate with the rate of enzymatic degradation of PRPP.

12.2. ALLOPURINOL AND THE METABOLISM OF OROTIC ACID

The enzyme complex formed by the two terminal enzymes of pyrimidine biosynthesis de novo, OPRT and ODC, catalyzes the conversion of orotic acid via OMP to UMP. The first enzyme of pyrimidine biosynthesis, carbamoylphosphate synthetase II, is usually regarded as the rate-limiting enzyme, but under certain conditions and in some cell types the conversion of orotic acid to OMP may be the limiting step of the de novo pathway (see 1.3). Deficiency of ADA or PNP may limit the availability of PRPP and in this way inhibit the further metabolism of orotic acid (see 1.5). The effects of allopurinol on OPRT and ODC will be discussed in detail below.

OPRT and ODC activities were measured in hemolysates of ten mammalian species (chapter 2). The enzyme assays are based on the release of $^{14}CO_2$ from [carboxyl-^{14}C]orotic acid or [carboxyl-^{14}C]orotidine 5'-monophosphate, respectively. The activities of both enzymes appeared to be very low in sheep hemolysates and below detection limit in horse hemolysates. OPRT and ODC showed a coordinate relationship in mammalian hemolysates, ODC activity being about twice as high as OPRT activity. Only pig and rabbit hemolysates showed a deviating ODC/OPRT ratio.

Sensitive and specific methods are needed in order to study the effects of allopurinol on the urinary excretion of orotic acid and orotidine. In chapter 6 an isotope dilution assay for orotic acid is described, which is based on the competition with a known amount of [carboxyl-^{14}C]orotic acid for reaction with a limiting amount of PRPP in the presence of purified OPRT and ODC. The dilution of the specific radioactivity of the $^{14}CO_2$ re-

leased is a measure of the amount of orotic acid present in the sample. Orotidine can be determined after hydrolytic cleavage to orotic acid. The excretion of orotidine was increased in all patients receiving allopurinol. After allopurinol administration orotic acid excretion was increased in gouty patients but close to normal values in patients with deficiency of HGPRT.

The increase in the urinary excretion of orotic acid and/or orotidine after allopurinol administration indicates an inhibition of OPRT and/or ODC activities. The mechanism of this inhibition is studied in chapter 7. When allopurinol or oxipurinol was incubated with hemolysate in the presence of PRPP, compounds were formed which strongly inhibited ODC activity. The inhibitors which were competitive with respect to OMP were presumably allopurinol ribonucleotide and oxipurinol 7-ribonucleotide (see Fig.1.7 for formulae). The inhibition of ODC leads to an accumulation of OMP, which may be dephosphorylated and excreted as orotidine in the urine. Furthermore, OMP proved to be an inhibitor of OPRT. It is obvious that the $^{14}CO_2$-method mentioned above could not be used to study the inhibition by OMP because this method depends on the ODC activity. Instead, [6-^{14}C]orotic acid was used as substrate and products were separated from unreacted orotic acid by thin-layer chromatography. The inhibition by OMP was competitive with respect to PRPP with a K_i value of 11 μM. The inhibition of OPRT by accumulated OMP might explain the increased urinary excretion of orotic acid after allopurinol administration. The absence of orotic aciduria after administration of allopurinol in patients with a deficiency of HGPRT is still consistent with the proposed mechanism, since the inhibition by OMP is competitive with PRPP and intracellular PRPP concentration is increased in these patients (see chapter 3). The same mechanism may also be responsible for the increased urinary excretion of orotic acid following administration of azauridine, azacytidine and azaorotic acid (see 6.5).

OPRT and ODC activities in hemolysates of allopurinol-treated patients are consistently higher than in control hemolysates, as reported in chapter 7. This phenomenon can not be ascribed to stabilization by allopurinol of these enzymes during cell lysis and extraction, since OPRT activity of controls is the same in hemolysates as in intact erythrocytes. During incubation of hemolysates at 37 °C, the activities of OPRT and/or ODC appeared to be stabilized by allopurinol ribonucleotide and oxipurinol 7-ribonucleotide, and also by PRPP and OMP. These in vitro experiments suggest that the apparent increase of OPRT and ODC activities after allopurinol therapy is

due to stabilization of the enzymes during the life-span of the erythrocytes The absence of a striking increase of OPRT and ODC activities in leukocytes after allopurinol administration is consistent with the theory of enzyme stabilization because of the short life-span of these cells.

Chapter 8 supplies evidence for the instability of OPRT and ODC in aging erythrocytes The activities of OPRT and ODC in erythrocytes from adults are much lower than in erythrocytes from newborns or from patients with reticulocytosis. In these latter two groups the mean red cell age is younger than in adults. Another factor which may contribute to the increased OPRT and ODC activities in neonatal erythrocytes is stabilization of the enzymes by PRPP which is present at a higher concentration in erythrocytes of newborns than in those of adults

Chapter 8 also deals with the activity of pyrimidine 5'-nucleotidase in erythrocytes of newborns and adults. This enzyme catalyzes the dephosphorylation of UMP, which is the product of the consecutive action of OPRT and ODC but also a degradation product of ribonucleic acid Pyrimidine 5'-nucleotidase was assayed by a new radiochemical method involving thin-layer chromatography. The activity of this enzyme was similar in erythrocytes of newborns and adults, and was higher with OMP as substrate than with UMP. Although OMP is a substrate for both ODC and pyrimidine 5'-nucleotidase, there is no mutual interference in the assays of both enzymes at appropriate substrate concentrations.

Several purine and pyrimidine nucleotides appeared to inhibit OPRT activity (chapter 7). The inhibition was competitive with respect to PRPP for all inhibitors tested, except TTP. Prior to testing the effects of nucleotides on OPRT activity their non-interference with the ODC reaction was ascertained (at 0.1 mM OMP) since the OPRT assay was dependent on ODC activity. During the OPRT assay, however, OMP concentration is very low and there might be a significant inhibition of ODC by nucleotides. Therefore we investigated in detail the inhibition of ODC activity in human hemolysates by purine and pyrimidine nucleotides at low concentrations of OMP (chapter 9). ODC of human hemolysate exhibited triphasic kinetics (chapters 7 and 9). The inhibition constants of all purine and pyrimidine nucleotides - except for CMP - with respect to ODC were high and inhibition of ODC could not be the cause of the decrease in $^{14}CO_2$ production from [carboxyl-^{14}C]orotic acid, which is observed when OPRT is assayed in the presence of these nucleotides. Inhibition of OPRT which is competitive

with respect to PRPP may be important for the regulation of pyrimidine biosynthesis and deserves further investigation.

12.3. ADENOSINE DEAMINASE DEFICIENCY AND PYRIMIDINE METABOLISM

Deficiencies of ADA and PNP in man are associated with certain forms of severe immunodeficiency. Although the precise mechanism which relates the enzyme deficiency to the immunodeficiency is not known, there is ample evidence that ADA deficiency causes an interference with pyrimidine biosynthesis de novo (see 1.5). It is difficult to obtain ADA-deficient lymphocytes for investigation since the deficiency is rare and the few patients known have severe lymphopenia. Therefore, many studies in this field are performed with model systems containing an inhibitor of ADA activity, e.g. EHNA (see 1.5). One objection to such studies is that the enzyme inhibitors are usually not quite specific. It seemed worthwhile to search for lymphocytes which by nature have a low activity of ADA. Since the absence of ADA activity in horse erythrocytes had been reported in the literature, we decided to measure ADA and PNP activities in erythrocytes and lymphocytes of the horse.

We could confirm the deficiency of ADA in horse erythrocytes (chapter 10). PNP activity was also virtually absent in these cells. The deficiency of both enzymes prevents the irreversible loss of purine nucleosides which would otherwise occur since HGPRT activity is absent in these cells (see chapter 2).

Both ADA and PNP activities were consistently lower (about 50%) in intact lymphocytes than in sonicated lymphocytes (chapter 10) suggesting that the uptake of nucleosides is rate-limiting. ADA activity in extracts of horse lymphocytes was much lower than in those of human lymphocytes and was comparable to the reported values for lymphocyte extracts of patients with severe combined immunodeficiency disease associated with ADA deficiency. Similar conclusions were reached in experiments with intact lymphocytes. ADA activity is apparently not necessary for normal lymphocyte function in horses. Comparison of lymphocyte metabolism in man and horse may provide valuable information about the role of ADA in lymphocytes. PNP activity in horse and cattle lymphocytes was about 20% of that in human lymphocytes.

Chapter 11 deals with comparative measurements of uridine metabolism

in erythrocytes and lymphocytes of man and horse Uridine was not metabolized in erythrocytes of both species The absence of uridine kinase in horse erythrocytes is remarkable since the enzymes OPRT and ODC are also absent (see chapter 2) and therefore UMP can also not be synthesized from exogenous orotic acid. Uridine nucleotides must be either conserved during the lifespan of the erythrocyte or supplied by some yet unknown pathway. Preliminary experiments in our laboratory indicate that the activity of pyrimidine 5'-nucleotidase is lower in horse erythrocytes than in human erythrocytes.

In lymphocyte extracts of horse the conversion of uridine to UMP proceeded more rapidly than the conversion to uracil. In lymphocyte extracts of man and cattle the catabolism of uridine to uracil appeared to be the major reaction of uridine metabolism. This conclusion was confirmed in experiments with intact lymphocytes. The metabolism of uridine in intact cells occurred at a much slower rate than in lymphocyte extracts, probably because nucleoside uptake is rate-limiting (cf. adenosine and inosine metabolism, chapter 10). Furthermore, ODC activity was markedly lower in lymphocyte extracts of horse than in those of man and cattle Horse lymphocytes apparently have a preference for the utilization of uridine to synthesize pyrimidine nucleotides An inhibition of pyrimidine synthesis de novo, associated with deficiency of ADA, may be bypassed by means of the salvage pathway.

It is interesting to compare the inhibition of pyrimidine synthesis de novo caused by deficiency of ADA with the hereditary deficiency of the enzymes OPRT and ODC. Accumulation of orotic acid was observed when cells were cultured in the presence of adenosine and ADA inhibitors, but there is no orotic aciduria in ADA- deficient patients. ADA deficiency apparently affects only some cell types, particularly the cells of the immune system. Most patients with hereditary orotic aciduria also have a disturbance of the immune system as evidenced by the occurrence of leukopenia. The megaloblastic anemia associated with the inherited deficiency of OPRT and ODC, however, is not found in patients with ADA deficiency. Uridine therapy is successful in hereditary orotic aciduria but seems to have little effect in patients with deficiency of ADA This discrepancy might be related to differences in the metabolism of uridine between various cell types. In human lymphocytes, which would be the target cells for uridine therapy in ADA deficiency, catabolism to uracil appears to be the major route of uridine metabolism.

Pyrimidine en purine nucleotiden spelen een belangrijke rol in de stofwisseling van de cel, onder andere als bouwstenen voor de nucleïnezuren en als co-enzymen. Deze nucleotiden kunnen op een tweetal manieren worden gevormd in de cel. Bij synthese "de novo" wordt uitgegaan van eenvoudige verbindingen als CO_2, ATP, glutamine en ribose in de vorm van phosphoribosylpyrophosphaat (PRPP). Bij de zogenaamde "salvage pathway" worden de nucleotiden gevormd uit stikstofbasen of nucleosiden. De enzymen van de "salvage pathway" zijn ook chemotherapeutisch van belang omdat een groot aantal antimetabolieten door deze enzymen wordt omgezet tot de werkzame verbindingen. Er zijn een aantal erfelijke stofwisselingsziekten bekend op het gebied van de pyrimidine- en purinestofwisseling. Deze vormden het uitgangspunt voor het hier beschreven onderzoek.

Pyrimidinestofwisseling en purinestofwisseling staan niet op zichzelf maar zijn in voortdurende wisselwerking met elkaar en met de stofwisseling van koolhydraten en aminozuren. De nauwe samenhang tussen pyrimidine- en purinestofwisseling is het belangrijkste thema van dit proefschrift. Een drietal aspekten van deze samenhang werd bestudeerd. de rol van PRPP in de stofwisseling van pyrimidines en purines (hoofdstukken 2-5), de effekten van allopurinol op de purine- en pyrimidinestofwisseling (hoofdstukken 6-9) en de mogelijke stoornis in de synthese van pyrimidine nucleotiden als gevolg van bepaalde erfelijke enzymdefekten in de purinestofwisseling (hoofdstukken 10-11).

Bij het bestuderen van de stofwisseling en de regulering ervan wordt veelal gebruik gemaakt van celextrakten of gezuiverde enzymen. Grote voorzichtigheid is geboden bij het extrapoleren van de resultaten van dergelijke studies naar het metabolisme van de intakte cel, omdat de kondities bij in vitro experimenten meestal sterk afwijken van de fysiologische kondities. Bovendien moet men er rekening mee houden dat tussen verschillende celtypes aanmerkelijke verschillen kunnen bestaan wat betreft de relatieve betekenis van bepaalde metabole routes. Daarom zijn in dit proefschrift, waar mogelijk, de resultaten verkregen met celextrakten vergeleken met metingen aan intakte cellen en zijn veelal zowel erythrocyten als lymphocyten betrokken in het onderzoek.

In hoofdstuk 1 wordt een overzicht gegeven van de huidige kennis omtrent de pyrimidine- en purinestofwisseling, de regulering ervan en de stoornissen die worden veroorzaakt door erfelijke enzymdeficiënties.

In hoofdstuk 2 worden de aktiviteiten gerapporteerd van de phosphoribosyltransferasen (adenine phosphoribosyltransferase, hypoxanthine-guanine phosphoribosyltransferase en orootzuur phosphoribosyltransferase) in hemolysaten van 10 zoogdierspecies. Beide laatstgenoemde enzymen waren afwezig in hemolysaat van het paard. De aktiviteit van orotidine-5'-monophosphaat decarboxylase bleek in hemolysaten van zoogdieren ongeveer tweemaal zo hoog als de aktiviteit van orootzuur phosphoribosyltransferase.

In hoofdstuk 3 worden de methodes beschreven die zijn ontwikkeld om de concentratie van PRPP en de snelheid waarmee deze verbinding wordt gevormd te bepalen. Verhoogde concentraties van PRPP werden gemeten in de erythrocyten van patienten met een deficientie van hypoxanthine-guanine phosphoribosyltransferase of van purine nucleoside phosphorylase. Deze verhoogde concentraties lijken een gevolg te zijn van een verminderd verbruik van PRPP.

Een verhoogde concentratie van PRPP kan ook worden veroorzaakt door een verhoogde synthesesnelheid. Een verhoogd aanbod van PRPP kan een versnelde purine biosynthese en een overproduktie van urinezuur teweegbrengen. In hoofdstuk 4 wordt het onderzoek beschreven naar de concentratie van PRPP en de aktiviteit van PRPP synthetase in erythrocyten van jichtpatienten en van een groep adolescenten met een hoge concentratie urinezuur in het serum. De aktiviteit van PRPP synthetase werd ook gemeten bij suboptimale concentraties van de substraten om afwijkingen in de substraataffiniteit van het enzym te kunnen ontdekken. Dergelijke kinetische varianten werden niet gevonden. De concentratie van PRPP in de erythrocyten was eveneens normaal. De aktiviteit van PRPP synthetase in hemolysaat was in de groep van adolescenten met een hoge urinezuurconcentratie in het serum significant hoger dan in de controlegroep. Deze bevinding kan van belang zijn voor het begrip van de pathogenese van primaire jicht.

In hoofdstuk 5 wordt de concentratie van PRPP in hemolysaten van tien zoogdierspecies vergeleken met de capaciteiten van de enzymen die betrokken zijn bij de synthese en het verbruik van deze verbinding. Een lage aktiviteit van hypoxanthine-guanine phosphoribosyltransferase ging steeds gepaard met een hoge concentratie van PRPP. In hoofdstuk 5 wordt tevens gerapporteerd over de enzymatische afbraak van PRPP in hemolysaten die veroorzaakt lijkt te worden door zure fosfatase.

In hoofdstuk 6 is een isotopenverdunningsmethode beschreven voor de bepaling van orootzuur, een intermediair uit de pyrimidine synthese de novo. Ook orotidine (dat ontstaat door dephosphorylering van orotidine-5'-monophosphaat) kan op deze manier worden bepaald na hydrolytische omzetting tot orootzuur. De uitscheiding van orotidine in de urine bleek verhoogd te zijn bij alle patienten die allopurinol kregen toegediend. Allopurinol is een structureel analogon van hypoxanthine en wordt therapeutisch gebruikt om de concentratie van urinezuur in het serum te verlagen (via remming van het enzym xanthine oxidase). De uitscheiding in de urine van orootzuur was bij de meeste patienten eveneens verhoogd na toediening van allopurinol.

Hoofdstuk 7 handelt over het mechanisme waarmee allopurinol een verhoogde excretie van orootzuur en orotidine in de urine kan teweegbrengen. Metabolieten van allopurinol (allopurinol ribonucleotide, oxipurinol-7-ribonucleotide) bleken competitieve remmers te zijn van orotidine-5'-monophosphaat decarboxylase. Hierdoor treedt een ophoping van orotidine-5'-monophosphaat op, dat in de vorm van orotidine kan worden uitgescheiden. Bovendien bleek orotidine-5'-monophosphaat een remmer te zijn van het enzym orootzuur phosphoribosyltransferase. De remming was competitief met PRPP. Deze remming kan een verklaring geven voor de verhoogde uitscheiding van orootzuur na toediening van allopurinol.

In hoofdstuk 7 is eveneens beschreven dat bij patienten met allopurinol-therapie de activiteiten van orootzuur phosphoribosyltransferase en orotidine-5'-monophosphaat decarboxylase in de erythrocyten verhoogd zijn. De resultaten van in vitro experimenten suggereren dat deze verhoogde enzymaktiviteiten mogelijk het gevolg zijn van stabilisatie van deze enzymen door metabolieten van allopurinol gedurende de levensduur van de erythrocyt. Een dergelijke verhoging van enzymaktiviteiten werd niet gevonden in leukocytenextracten.

De stabiliteit van enzymen van de pyrimidinestofwisseling in de ouderwordende erythrocyt vormt het belangrijkste onderwerp van hoofdstuk 8. De aktiviteiten van orootzuur phosphoribosyltransferase en orotidine-5'-monophosphaat decarboxylase bleken lager te zijn in erythrocyten van volwassenen dan in rode bloedcellen van pasgeborenen, welke gemiddeld een jongere celleeftijd hebben. De aktiviteit van pyrimidine-5'-nucleotidase bleek hetzelfde te zijn in erythrocyten van pasgeborenen en van volwassenen. Dit enzym werd gemeten met een nieuwe radiochemische methode en vertoonde een hogere aktiviteit met orotidine-5'-monophosphaat dan met uridine-5'-

monophosphaat als substraat.

Hoofdstuk 9 handelt over de invloed van purine en pyrimidine nucleotiden op de activiteiten van orootzuur phosphoribosyltransferase en orotidine-5'-monophosphaat decarboxylase in hemolysaten van de mens. Het laatstgenoemde enzym vertoonde trifasische kinetiek. Enige nucleotiden bleken remmers te zijn van orootzuur phosphoribosyltransferase (veelal competitief ten opzichte van PRPP) of van orotidine-5'-monophosphaat decarboxylase (competitief met orotidine-5'-monophosphaat).

In hoofdstuk 10 wordt gerapporteerd over de activiteiten van adenosine deaminase en purine nucleoside phosphorylase in lymfocyten en hemolysaten van mens, paard en rund. Deficienties van deze enzymen gaan bij de mens gepaard met stoornissen in de cellulaire en humorale immuniteit. De activiteiten van beide enzymen waren in intakte lymfocyten steeds lager dan in lymfocytenextracten. De aktiviteit van adenosine deaminase bleek zeer laag te zijn in zowel hemolysaten als in lymfocyten van het paard.

Aangezien deficientie van adenosine deaminase bij de mens gepaard schijnt te gaan met een remming van de pyrimidine synthese "de novo", werd een onderzoek gedaan naar het relatieve belang van de "salvage pathway" voor de synthese van pyrimidine nucleotiden in lymfocyten en erythrocyten van mens en paard. De resultaten van dit onderzoek zijn beschreven in hoofdstuk 11. Uridine bleek niet te worden omgezet door erythrocyten. De omzettingssnelheid van uridine in intakte lymfocyten was veel geringer dan die in extracten van lymfocyten. De activiteit van uridine kinase was vergelijkbaar in lymfocyten van mens, rund en paard. Een hoge activiteit van uridine fosforylase werd gemeten in lymfocytenextracten van mens en rund maar niet in die van het paard. Intakte lymfocyten van de mens maar niet die van het paard bleken in staat om uridine om te zetten tot uracil. Bovendien was de aktiviteit van orotidine-5'-monophosphaat decarboxylase (het laatste enzym van de "de novo" synthese) veel lager in lymfocytenextracten van het paard dan in die van de mens en het rund. Lymfocyten van het paard schijnen een voorkeur te hebben voor het gebruik van uridine om pyrimidine nucleotiden te synthetiseren.

In hoofdstuk 12 tenslotte is getracht de belangrijkste resultaten van de voorafgaande hoofdstukken te kombineren en hun onderlinge samenhang te verduidelijken.

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   Activity of purine phosphoribosyltransferase and of two enzymes of pyrimidine biosynthesis in erythrocytes of ten mammalian species.
   Comp.Biochem.Physiol. 54B, 209-212.
2. Tax, W.J.M. and Veerkamp, J.H. (1977)
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   Clin.Chim.Acta 78, 209-216.
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5. Tax, W.J.M., Veerkamp, J.H., Trijbels, J.M.F. and Schretlen, E.D A.M. (1976)
   Mechanism of allopurinol-mediated inhibition and stabilization of human orotate phosphoribosyltransferase and orotidine phosphate decarboxylase.
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   Pyrimidine metabolism in erythrocytes of the newborn.
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7. Tax, W.J.M. and Veerkamp, J.H.
   Inhibition of orotate phosphoribosyltransferase and orotidine 5'-phosphate decarboxylase of human erythrocytes by purine and pyrimidine nucleotides.
   Biochem.Pharmacol. (in press).
8. Tax , W.J.M. and Veerkamp, J.H.
   Activity of adenosine deaminase and purine nucleoside phosphorylase in erythrocytes and lymphocytes of man, horse and cattle.
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Mixed acylals; synthesis of alkylidene carboxylate formates.
Synthesis 1973, 151-153.

CURRICULUM VITAE

Wil J.M. Tax werd geboren in Groesbeek op 29 september 1949.
Hij behaalde het diploma Gymnasium B in 1967 (Gabriël College, Mook) en begon in hetzelfde jaar met de scheikunde studie aan de Katholieke Universiteit te Nijmegen. Het kandidaatsexamen (S 2) werd afgelegd in juli 1970 en het doctoraaalexamen in september 1973 met als hoofdrichting biochemie (Dr. J.H.Veerkamp) en als bijvakken organische chemie (Prof.Dr. R.J.F. Nivard) en farmacologie (Prof.Dr. J.M. van Rossum). Vervolgens werd op de afdeling Biochemie van voornoemde universiteit het hier beschreven onderzoek verricht onder leiding van Dr. J.H.Veerkamp en Prof.Dr. E.D.A.M.Schretlen (afdeling Kindergeneeskunde). Tevens werd een bijdrage geleverd aan het onderwijs aan pre-kandidaats studenten in de geneeskunde en de tandheelkunde en aan doctoraalstudenten scheikunde en biologie. Vanaf 1 februari 1975 was hij in dienst van de Nederlandse Organisatie voor Zuiver-Wetenschappelijk Onderzoek (Z.W.O.) via de Stichting Fundamenteel Geneeskundig Onderzoek (FUNGO).

In 1976 is hij getrouwd met Marijke Schraven.

T O E L I C H T I N G

Gewaardeerde leek,

Wanneer U geheel onbekend bent met de biochemie, zal het U wellicht moeilijk vallen om te begrijpen waarover dit proefschrift handelt. Misschien begint U verwachtingsvol de Nederlandstalige samenvatting te lezen maar raakt U de draad kwijt bij termen als pyrimidines en purines, enzymen en metabolieten, stofwisselingsziekten. Daarom wil ik proberen in het kort toe te lichten wat de achtergronden zijn van het hier beschreven onderzoek.

In de cellen van alle levende wezens vindt voortdurend een groot aantal scheikundige reakties plaats waarbij stoffen worden gevormd of afgebroken. Deze omzettingen worden teweeggebracht door bepaalde eiwitten, enzymen genaamd. Voor elke reaktie is een apart enzym aanwezig in de cel. Het geheel van al deze omzettingen noemen we de stofwisseling of het metabolisme. De verbindingen die worden gevormd of afgebroken noemen we metabolieten. Omdat de stofwisseling erg veel verschillende reakties omvat is het ondoenlijk de gehele stofwisseling tegelijk te bestuderen. Veelal beperken onderzoekers zich dan ook tot een bepaald onderdeel, bv. de koolhydraatstofwisseling, de vetstofwisseling enz.

In de zogenaamde nucleïnezuren van de cel is de informatie vastgelegd die bepaalt welke eiwitten (waaronder de enzymen) door de cel zullen worden geproduceerd. Deze informatie wordt van geslacht tot geslacht overgeërfd. Een afwijking in dit erfelijke materiaal kan tot gevolg hebben dat in de cel een verkeerd eiwit wordt gemaakt, bv. een enzym dat niet werkzaam is. In dat geval spreekt men van een erfelijke stofwisselingsziekte. Van ongeveer 200 erfelijke stofwisselingsziekten is thans bekend welk eiwit abnormaal is. Een bekend voorbeeld is phenylketonurie (PKU). Alle pasgeborenen in Nederland worden tegenwoordig (met behulp van een hielprik) onderzocht op deze ziekte. Daarnaast is bij een aantal ziekten bekend dat erfelijke faktoren een rol spelen zonder dat precies is opgehelderd op welke manier. Voorbeelden zijn suikerziekte en jicht.

De bovengenoemde nucleïnezuren zijn o.a. opgebouwd uit de bouwstenen purines en pyrimidines. Ook in de stofwisseling van deze purines en pyrimidines zijn een aantal erfelijke afwijkingen bekend. Hiermee zijn we aange-

land op het terrein van onderzoek waarover dit proefschrift handelt. Onderzoek op het gebied van erfelijke stofwisselingsziekten is niet alleen van belang voor de betrokken patienten, maar kan ook leiden tot een beter begrip van het verloop en de regulering van de normale stofwisseling. Zo is het met name uit de bestudering van aangeboren afwijkingen duidelijk geworden dat er een sterke onderlinge samenhang bestaat tussen de pyrimidinestofwisseling en de purinestofwisseling. Deze onderlinge samenhang is vooral een gevolg van de regulerende rol van phosphoribosylpyrophosphaat, afgekort PRPP. De vele reakties die betrokken zijn bij de vorming en het verbruik van deze verbinding zijn weergegeven in Figuur 1.5 (proefschrift, pag. 9). In de eerste hoofdstukken van dit proefschrift zijn de resultaten beschreven van onderzoekingen met betrekking tot deze verbinding PRPP. Onder andere werd onderzocht of er in de rode bloedcellen van jichtpatiénten afwijkingen zijn in de stofwisseling van PRPP. Vervolgens worden experimenten beschreven over de bijwerkingen van het geneesmiddel allopurinol. Dit geneesmiddel wordt (o.a. bij jichtpatienten) gebruikt om afwijkingen in de purinestofwisseling te korrigeren, maar heeft ook bijwerkingen met betrekking tot de stofwisseling van pyrimidines, zoals blijkt bij onderzoek van de urine. De laatste hoofdstukken handelen over een tweetal erfelijke afwijkingen in de stofwisseling van de purines. Deze afwijkingen komen tot uiting in een gestoorde immunologische afweer en lijken veroorzaakt te worden door een storing in de stofwisseling van de pyrimidines.

Men zal zich afvragen wat al deze onderzoekingen nu hebben opgeleverd. Welnu, wie denkt dat de verschijnselen van de onderzochte erfelijke ziekten nu volledig verklaard kunnen worden of dat er misschien zelfs een geneeswijze kan worden aangegeven, komt bedrogen uit. Dit soort vraagstukken is meestal zo ingewikkeld dat ze alleen zijn op te lossen wanneer de gegevens die gedurende vele jaren zijn vergaard in talloze laboratoria over de gehele wereld worden gekombineerd. De hier beschreven onderzoekingen geven meer inzicht in de manier waarop allopurinol de stofwisseling van pyrimidines beïnvloedt. Verder is aangetoond dat sommige enzymen van de purine- en pyrimidinestofwisseling afwezig zijn in bloedcellen van bepaalde dieren. Bestudering van deze cellen kan misschien bijdragen tot een beter begrip van deze stofwisseling bij de mens. Bovendien zijn methoden beschreven om op een betrouwbare manier erg lage concentraties van PRPP en van orootzuur te meten. Mogelijk komen deze methoden andere onderzoekers van pas bij hun experimenten.

Nijmegen, november 1978. Wil Tax

STELLINGEN

I

De op isotopenverdunning gebaseerde bepaling voor orootzuur zoals beschreven door Cohen et al. is niet korrekt wegens een onjuiste verhouding tussen de concentraties van phosphoribosylpyrophosphaat en [carboxyl-^{14}C]orootzuur.
A.Cohen, G.E.J.Staal, A.J.Ammann en D.W.Martin (1977) J.Clin.Invest. 60. 491-494.
Dit proefschrift, hoofdstuk 6.

II

De konklusie van Janeway en Cha dat er een orotidine phosphorylase voorkomt in lymphoma-cellen wordt niet gerechtvaardigd door hun experimenten.
C.M.Janeway en S.Cha (1977) Cancer Res. 37, 4382-4388.

III

Beardmore et al. houden bij de interpretatie van hun gegevens onvoldoende rekening met de instabiliteit van phosphoribosylpyrophosphaat.
T.D.Beardmore, J.C.Meade en W.N.Kelley (1973) J.Lab.Clin.Med. 81, 43-52.
Dit proefschrift, hoofdstuk 8.

IV

De hypothese van Grobner en Kelley dat de verhoogde aktiviteiten van orootzuur phosphoribosyltransferase en orotidine-5'-phosphaat decarboxylase in hemolysaten na toediening van allopurinol een gevolg is van stabilisatie van deze enzymen gedurende de extractieprocedure, is onjuist.
W.Grobner en W.N.Kelley (1975) Biochem.Pharmacol. 24, 379-384.
Dit proefschrift, hoofdstuk 7.

V

De nummering van de pyrimidine-ring zoals die gehanteerd wordt door sommige leveranciers van radioaktieve verbindingen is niet in overeenstemming met de IUPAC-regels en werkt daardoor verwarrend.

VI

Het gebruik van strychnine als geneesmiddel bij niet-ketotische hyperglycinemie toont opnieuw aan dat het verschil tussen geneesmiddel en gif voornamelijk bepaald wordt door de dosis.
R.Gitzelmann, B.Steinmann en M.Cuenod (1978) N.Engl.J.Med. 298, 1424

VII

De snelheid waarmee de stofwisseling in zoogdieren plaatsvindt wordt in belangrijke mate bepaald door de snelheid waarmee het bloed substraten en zuurstof aanvoert.
R.A.Coulson, T.Hernandez en J.D.Herbert (1977) Comp.Biochem.Physiol. 56A, 251-262.

VIII

De opvatting dat de dalende perinatale sterfte een gevolg is van het stijgende percentage bevallingen in het ziekenhuis dient te worden betwijfeld.
G.J.Kloosterman (1978) Ned.T.Geneesk. 122, 1161-1171.

IX

Bij de bestudering van de samenhang tussen zoutconsumptie en hypertensie dient meer aandacht te worden besteed aan de hoeveelheid kalium in het dieet.
G.R.Meneely en H.D.Battarbee (1976) Am.J.Cardiol. 38, 768-785.

X

De term zwangerschapsonderbreking suggereert ten onrechte dat het hier een ingreep betreft die weer ongedaan gemaakt kan worden.

XI

Invoering van de bekende regel uit het straatvoetbal "driemaal hoekschop is strafschop" kan het voetbal als kijksport ten goede komen.

XII

Voor Amerikaanse toeristen kan binnenkort gelden: op de markt is Uw dollar een daalder waard.

Nijmegen, 17 november 1978 W.J.M. Tax